Docket No.: 20555/1203301-US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Daniel G. Chain

Application No.: 10/084,380 | Confirmation No.: 3496

Filed: February 28, 2002 | Art Unit: 1649

For: **SPECIFIC ANTIBODIES TO AMYLOID BETA PEPTIDE, PHARMACEUTICAL COMPOSITIONS AND METHODS OF USE THEREOF** | Examiner: G. S. Emch

SECOND DECLARATION OF KENNETH L. ROCK, M.D. UNDER 37 C.F.R. §1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Kenneth L. Rock declares and states as follows:

1. I am a citizen of the United States, more than twenty-one years of age, and make this Second Declaration in support of the above-identified application.

2. I have previously made a Declaration Under 37 C.F.R. §1.132 in support of this application. The contents of my previous Declaration are hereby incorporated herein by reference.

3. I participated in a personal interview between the Applicant and his representatives and the examiners responsible for the application that was held at the Patent and Trademark Office on April 23, 2008 at which the attendees (myself, the inventor, Dr. Chain, Examiners Emch, Kemmerer and Stucker, Applicant's patent counsel, Mr. Ludwig and Dr.

Bernstein, and Dr. Federoff) discussed the Office Action in the application that was on mailed November 19, 2007.

4. During the April 23 interview, I indicated that by the filing date of provisional application 60/041,850 ("the provisional application") on April 9, 1997, it was known that antibodies crossed the blood brain barrier. The Examiners requested that references be provided on this point. The references submitted with this Declaration establish that by April 1997 it was known that naturally occurring antibodies and antibodies administered intravenously crossed the brain barrier and were present in cerebrospinal fluid (CSF).

5. Sherwin et al., *Neurology* 13:113-119 (1963) (attached at Tab A) demonstrated that antibodies to bovine serum albumin (BSA) were present in the CSF of rabbits, following either active immunization or transfer of antibody administered intravenously. Passage of antibody from the serum to the CSF following passive immunization was increased in animals in which inflammation of the central nervous system (CNS) was present due to acute experimental encephalomyelitis.

6. Fossan, G. O., *Eur. Neurol.* 15:231-236 (1977) (attached at Tab B) reported that naturally occurring IgG antibodies to rabbit erythrocytes (RRBC) are present in human CSF. IgG levels in the CSF relative to serum IgG levels increased in patients with subacute sclerosing panencephalitis, inflammatory diseases, and certain diseases of the spine.

7. Elovaara et al., *Eur. Neurol.* 26:229-234 (1987) (attached at Tab C) studied antibody levels in the CSF of normal individuals and patients with Alzheimer's disease and multi-infarct dementia (MID). Antibodies were found in the CSF of normal individuals and patients with Alzheimer's disease and MID, with IgG being present in higher concentrations than either IgA or IgM. According to Elovaara, the permeability of the blood brain barrier to immunoglobulins was increased in patients with both Alzheimer's disease and MID.

8. Kalofonos et al., *J. Nucl. Med.* 30:1636-1645 (1989) (attached at Tab D) found that ^{123}I-radiolabeled IgG to epidermal growth factor receptor or placental alkaline phosphatase localized to tumors in 18 of 27 glioma patients, when administered either intravenously or via the

internal carotid artery. Both specific and non-specific localization to tumor was observed. Ten relapsed patients who had demonstrated good tumor localization of ^{123}I-radiolabeled antibody were treated with ^{131}I-labeled antibody. Six of the ten treated patients exhibited clinical improvement over a period lasting from six months to three years.

9. Zlokovic et al., *Neurology* 107:263-270 (1990) (attached at Tab E) reported the presence in guinea pigs of a specific, saturable and unidirectional pathway for transport of IgG from the serum to the CSF.

10. Aihara et al., *J. Comp. Neurol.* 342:481-496 (1994) (attached at Tab F) used immunolocalization techniques at the light and ultrastructural levels to demonstrate that IgG crossed the blood-brain barrier of rats. Increased levels of IgG were found in the brain following head injury.

11. The references discussed above establish that by April 1997, one of ordinary skill in the art would have appreciated that antibodies, particularly IgG, cross the blood brain barrier, that conditions that lead to inflammation or other injury of the brain generally increase the levels of antibodies that cross the blood brain barrier, that patients with Alzheimer's disease, in particular, are likely to have increased levels of IgG in the CSF, and that antibodies that are administered intravenously can have therapeutic effects.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant's signature:

/Kenneth L. Rock/ May 19, 2008

Kenneth L. Rock, M.D. Date

TAB A

to
Second Declaration
of Kenneth L. Rock, M.D.,
dated May 19, 2008

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Studies of the blood-cerebrospinal fluid barrier to antibodies and other proteins

A. L. Sherwin, M.D., M. Richter, Ph.D.,
J. B. R. Cosgrove, M.D., and B. Rose, M.D.

EARLY INTEREST in the blood–cerebrospinal fluid barrier to antibody was stimulated by the use of immune horse serum in the treatment of meningitis. Ransom[1] observed the presence of tetanus antitoxin in the cerebrospinal fluid (CSF) of a horse with a high serum antitoxin titer, and the conclusion drawn from this and various other studies was that antibody molecules were capable of penetrating the blood-CSF barrier.[2,3] However, there has been little agreement as to the circumstances under which any particular antibody could be found in the CSF. In general, the antigens used to stimulate antibody production were not homogeneous but were bacteria or red blood cells which are now known to give rise to antibodies which differ considerably in molecular weight. Furthermore, the methods employed for the detection of antibodies were not as sensitive as those available today. Interest in the blood-CSF barrier, in relation to antibody penetration, diminished with the advent of chemotherapy. However, the recent demonstration of antibodies to autologous tissues in various illnesses characterized as "auto-immune" diseases (such as thyroiditis, rheumatoid arthritis, and lupus erythematosis) has sparked similar investigations of various diseases of the nervous system. Postinfectious encephalitis, Guillain-Barré syndrome, and some of the demyelinating diseases particularly have been suspected of being the results of abnormal immunologic mechanisms. Since it is generally acknowledged that antibodies are formed in the reticuloendothelial tissues (that is, the spleen and lymph nodes), it follows that they must first penetrate the blood-brain or blood-CSF barrier, or both, if they are to produce lesions in the nervous tissue. Antibodies to horse serum,[4] the reagin in syphilis,[5] anti-streptolysin-O,[6] and auto-antibodies to thyroid[7] have been demonstrated in the CSF.

The object of this study was to re-evaluate, by the use of pure protein antigens and sensitive immunochemical technics, the quantitative relationship of antibodies in the serum and the CSF and to determine the rate at which antibody and gamma globulin molecules can penetrate the blood-CSF barrier.

MATERIALS AND METHODS

Albino female rabbits, weighing an average of 3 kg., were employed for these experiments.

Active immunization was carried out by the intravenous administration of 15 mg. of antigen 3 times a week for three weeks. The animals were divided into 3 groups according to the antigen administered. Group I received bovine serum albumin (BSA), group II was injected with ovalbumin (OA), and group III received a combination of the 2 antigens. The rabbits were bled through the marginal ear vein eight to twelve days after the last injection. The antisera obtained in this manner were Seitz filtered, merthiolate was added to

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Dr. Sherwin is a John and Mary R. Markle Scholar in Medical Science in the Department of Neurology and Neurosurgery, McGill University.

a concentration of 1:10,000, and aliquots were frozen in sterile vials and stored at –20° C.

In order to measure the rate of entry of proteins from the blood into the CSF, rabbits were injected intravenously with anti-BSA or a mixture of anti-BSA and anti-OA, or with BSA. To measure the rate of entry of proteins from the CSF into the blood, cisternal puncture was performed in other animals, and 0.5 ml. of a 5 per cent solution of BSA was injected after removal of an equal volume of CSF.

Cisternal puncture was performed with sterile technic following sedation with pentobarbital sodium (Nembutal). A short, 20-gauge spinal puncture needle was used and the CSF was withdrawn slowly in several small aliquots. All specimens of CSF were examined microscopically and those with evidence of contamination with blood were discarded. CSF volumes varied from 0.5 to 1.5 ml. In cases where a second puncture was planned on the same animal, only 0.5 ml. were initially removed. Animals were bled from the marginal ear vein just before cisternal puncture; the blood was permitted to clot and was then separated in the usual manner. The sera and corresponding CSF were frozen and stored at –20° C. Rabbits from which clear CSF was obtained were sacrificed and the brain and spinal cord removed for histologic study. Those displaying evidence of encephalitis were eliminated from the experiment.

Experimental allergic encephalomyelitis was produced in rabbits by a single injection of rabbit spinal cord in Freund's adjuvant, as previously described.[8] At the time of overt clinical symptoms, anti-BSA (rabbit) serum was administered intravenously (4 ml. per kilogram of body weight). Blood and CSF were sampled twenty-four-hours later.

Antibody titers were determined by means of the BDB hemagglutination technic.[9] By this technic, antigen is coupled, irreversibly, to red blood cells by the use of bisdiazotized benzidine (BDB). Such "sensitized cells" are

TABLE 1

COMPARISON OF ANTIBODY TITERS IN SERUM AND CSF OF NORMAL RABBITS FOLLOWING ACTIVE IMMUNIZATION WITH BOVINE SERUM ALBUMIN (BSA) AND OVALBUMIN (OA)

Rabbit no.	*Antigen used*	*BDB titer**		*Ratio serum/CSF*
		Serum	*CSF†*	
1	BSA	256,000	640	400
2	BSA	512,000	1,280	400
3	BSA	1,024,000	640	1,600
4	BSA	256,000	320	800
5	BSA	512,000	1,280	400
6	BSA	64,000	80	800
	OA	1,024,000	640	1,600
7	BSA	32,000	80	400
	OA	512,000	1,280	400
8	BSA	2,000	20	100
	OA	256,000	640	400
9	BSA	128,000	80	1,600
	OA	512,000	640	800
10	BSA	64,000	40	1,600
	OA	256,000	80	3,200
11	BSA	1,000	<10‡	—
	OA	128,000	20	6,400

*Antibodies measured by bisdiazotized benzidine hemagglutination technic (BDB)
†Cisternal puncture immediately following bleeding from ear vein
‡Titers of less than 10 are considered negative.

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RESULTS

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capable of being subsequently agglutinated by the respective antibodies. Serial halving dilutions of the serum or CSF to be tested are prepared in buffered saline and an aliquot of "sensitized cells" is added to each tube. In the presence of antibody, hemagglutination is observed. Determinations of the amount of BSA in the serum or CSF were carried out by means of the BDB inhibition technic, using known quantities of BSA as standards to inhibit the agglutination of BSA-sensitized red cells by anti-BSA. In order to minimize experimental variation, the serum and CSF specimens from each animal were assayed simultaneously.

RESULTS

Comparison of antibody titers in serum and CSF following active immunization. Antibodies were detected in samples of CSF obtained from each of 11 rabbits fourteen days after the last of a series of 9 intravenous injections of antigen (table 1). Antibodies to both BSA and OA were present in the CSF of 5 out of 6 rabbits which had been immunized with both BSA and OA simultaneously. In one rabbit, the serum titer of anti-BSA was only 1,000 and no antibody was detected in the CSF. Though the antibody titers in the CSF varied greatly, the relative distribution of antibody between serum and CSF was similar in individual animals. This has been expressed as the ratio of the serum titer to the CSF titer. In most of the animals the serum titer was found to be 400–800 times greater than that of the CSF titer.

Passage of antibodies from serum to CSF. These results are summarized in table 2. Normal rabbits were divided randomly into 6 groups, comprising a total of 18 animals. Each rabbit received an intravenous injection of immune rabbit serum (4 ml. per kilogram of body weight) obtained from other hyperimmunized rabbits. Samples of blood and CSF were obtained from the animals of each group at the following time intervals: one, one and one-half, four, twelve, and twenty-four hours, as well as seven days. No antibodies could be detected in the CSF samples obtained at one, one and one-half, or four hours, although the serum titers were high. Antibodies were detected in the CSF of 3 out of 4 rabbits sampled at twelve hours. However, the titers were very low in the CSF even though 2 of the rabbits had serum titers of over 1,000,000. Their serum/CSF ratios were 51,200 and 5,120. All 10 CSF samples obtained at twenty-four hours had detectable antibody. The serum/CSF ratios were now similar in magnitude to those of actively immunized rabbits. One animal (No. 26) received serum containing both anti-BSA and anti-OA. Although there was a great discrepancy in the serum antibody titers to the 2 antigens, the serum/CSF ratios were the same, indicating that both antibodies established a similar relative distribution across the blood-CSF barrier. Two animals were sacrificed after seven days (Nos. 24 and 30). The serum titers of both animals were 400,000 after twenty-four hours, decreasing to 100,000 at the end of seven days. In the one CSF tested, the titer had similarly dropped from 320 to 40 over this period. The decline in serum antibody titer is attributed to the half-life of serum gamma globulin, and the drop in the CSF titer appears to reflect the same factor.

Entry of anti-BSA antibodies into CSF of rabbits with experimental allergic encephalomyelitis. Five rabbits were injected with rabbit spinal cord in Freund's adjuvant. Two developed severe paralytic encephalomyelitis between fifteen and eighteen days, at which time they were given, intravenously, anti-BSA (rabbit) serum (4 ml. per kilogram of body weight). Blood and CSF samples were obtained from these 2 animals twenty-four hours later and microscopic examination of their CSF revealed a lymphocytic pleocytosis. The anti-BSA titers in both samples of CSF were higher than in any of the other 23 specimens obtained from similarly injected normal rabbits (table 2). The serum/CSF ratio of both rabbits was 50, indicating that there was a relatively larger amount of antibody in the CSF. Histopathologic examination of the CNS of both rabbits revealed extensive perivascular inflammatory lesions in the brain and spinal cord.

Passage of bovine serum albumin from blood to CSF. Seven rabbits were divided into 2 groups and each rabbit received a single intravenous injection of 1 ml. of a 5 per cent solution of BSA (50 mg.). Samples of serum and

TABLE 2

PASSAGE OF PASSIVELY TRANSFERRED HOMOLOGOUS ANTIBODIES TO BOVINE SERUM ALBUMIN FROM SERUM TO CSF IN NORMAL RABBITS AND THOSE WITH EXPERIMENTAL "ALLERGIC" ENCEPHALOMYELITIS (EAE)

Robbit no.	Histology of CNS	Time from I.V. injection to cisternal puncture and bleeding (hours)	BDB titer* Serum	BDB titer* CSF†	Ratio serum/CSF
13	Normal	1	32,000	< 10‡	—
14	Normal	1½	64,000	< 10	—
15	Normal	4	32,000	< 5	—
16	Normal	4	64,000	< 10	—
17	Normal	4	8,000	< 5	—
18	Normal	4	8,000	< 5	—
19	Normal	4	8,000	< 5	—
20	Normal	12	1,024,000	20	51,200
21	Normal	12	51,200	10	5,120
22	Normal	12	1,024,000	20	51,200
23	Normal	12	102,400	< 10	—
13	Normal	24	32,000	20	1,600
16	Normal	24	32,000	40	800
17	Normal	24	8,000	10	800
18	Normal	24	8,000	10	800
24	Normal	24	400,000	320	1,250
25	Normal	24	200,000	160	1,250
26	Normal	24	32,000	40	800
			4,096,000§	5,120§	800§
27	Normal	24	32,000	10	3,200
28	Normal	24	16,000	20	800
29	Normal	24	32,000	10	3,200
30	Normal	24	400,000	¶	¶
24	Normal	7 days	100,000	40	2,500
30	Normal	7 days	100,000	40	2,500
31	EAE	24	64,000	1,280	50
32	EAE	24	32,000	640	50

*Antibodies measured by bisdiazotized benzidine hemagglutination technic (BDB)
†Cisternal puncture immediately following bleeding from ear vein
‡Titers of less than 10 are considered negative.
§Injected with homologous antiovalbumin, in addition
¶Test was not performed.

CSF were obtained after four hours in 3 rabbits. BSA could be detected by ring test in the serum diluted fourfold, and levels of 20 to 63 μg. of BSA per milliliter were determined by the BDB inhibition technic (table 3). BSA could not be detected by ring test on the undiluted CSF nor by BDB inhibition, which was sufficiently sensitive to detect less than 0.02 μg. per milliliter. BSA was detected in the serum and CSF of all 4 rabbits which were sampled after twenty-four hours. The serum/CSF ratio varied from 133 to 1,575, which is in the same order of magnitude as that observed with the passively transferred antibodies (gamma globulin).

Passage of BSA from CSF to blood. Twenty-

PASSAGE OF BOVINE

Rabbit no.	Interval bet injection of BSA and c puncture
71	4
72	4
73	4
74	24
75	24
76	24
77	24

•Determined by inhibition of the
†Obtained from marginal ear vei
‡Positive interfacial ring test

five milligrams of BSA i
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TABLE 3

PASSAGE OF BOVINE SERUM ALBUMIN FROM BLOOD TO CSF IN NORMAL RABBITS

Rabbit no.	Interval between I.V. injection of 50 mg. BSA and cisternal puncture (hours)	Ring test		BSA concentration* γ/cc.		Ratio Serum/CSF
		Serum†	CSF	Serum†	CSF	
71	4	+‡	0	20	<.02	—
72	4	+	0	63	<.02	—
73	4	+	0	63	<.02	—
74	24	+	+	63	.04	1,575
75	24	+	+	20	.04	500
76	24	+	+	125	.08	1,562
77	24	+	+	16	.12	133

*Determined by inhibition of the BDB test
†Obtained from marginal ear vein just before cisternal puncture
‡Positive interfacial ring test

five milligrams of BSA in 0.5 ml. of sterile Elliott and Jasper's artificial CSF[10] were slowly injected into the cisterna magna of 2 rabbits after removal of an equal volume of clear CSF. Blood samples were taken at ten minutes and four hours following the injection (table 4). No BSA could be detected in the undiluted serum obtained at ten minutes by ring test nor by the BDB inhibition technic. However, both of the serum samples obtained at four hours gave positive interfacial ring tests even when diluted fourfold, and 6.4 μg. of BSA per cubic centimeter were detected by the BDB inhibition test. Both rabbits produced good antibody titers following immunization by this route. Further studies on immunization through the subarachnoid route are in progress.

DISCUSSION

Freund[2] measured agglutinins to S. typhosa in the blood and CSF of rabbits. The CSF was obtained after the animals had been completely exsanguinated under ether anesthesia. Histologic examinations were not done, as the brains were extracted to measure their antibody content. It is possible that a number of these animals may have had subclinical enzootic encephalitis, which is very prevalent in rabbits. As Freund noted, this factor could affect the blood-CSF barrier and alter the composition of the CSF. This study showed that the CSF antibody titers were about 1:300 of those of the sera, with maximal titers obtained about fifteen hours after intravenous injection of the antisera. Similar results were reported by Sohier and co-workers[3] who measured diphtheria and tetanus antitoxin in the blood and CSF of patients with craniofacial injuries. Arbesman and associates[4] reported a patient who developed Guillain-Barré syndrome following the administration of antitetanus serum. Employing the same technics as used in our studies, he found the serum/CSF ratio of the horse antitetanus antibodies to be 300 and that the CSF titer diminished proportionately to that of the blood. Hall and Owen,[7] employing Boyden's tanned-cell agglutination technic, found auto-antibodies to thyroid in the CSF of several patients with thyroiditis, the serum/CSF ratio averaging about 1,000. This distribution of antibody gamma globulin between blood and CSF is in agreement with the findings of Kabat and associates[11] who showed

TABLE 4

PASSAGE OF BOVINE SERUM ALBUMIN FROM CSF TO BLOOD FOLLOWING INTRACISTERNAL INJECTION IN NORMAL RABBITS

Rabbit no.	Interval between I.C.* injection and bleeding	Ring test serum‡	BSA concentration† γ/cc. serum
78	10 minutes	0	<0.01
	4 hours	+§	6.4
79	10 minutes	0	<0.01
	4 hours	+	6.4

*Intracisternal injection of 25 mg. of BSA in 0.5 cc. of sterile Elliott and Jasper's artificial CSF solution after removal of 0.5 cc. CSF
†As determined by inhibition of the BDB test
‡Obtained from marginal ear vein
§Positive interfacial ring test

that CSF gamma globulin appears to be largely derived from the blood. Starnes and others[12] were unable to demonstrate rheumatoid factor in the CSF.

The results of our experiments demonstrate that antibodies can be detected in the CSF of normal rabbits, after either active or passive immunization. The titer of an individual CSF specimen was found to be proportional to that of the homologous serum specimen and therefore reflected the relative distribution of gamma globulin across the blood-CSF barrier. Antibody appeared to enter the CSF slowly following intravenous administration and attained maximal levels by twenty-four hours. The CSF titer of the passively transferred antibody fell over a period of seven days to a degree similar to that of the serum titer. Removal of about half the rabbit CSF volume at intervals of one to four hours after the intravenous administration of antiserum did not appear to affect the titer observed in the second sample of CSF obtained after twenty-four hours. There was an increase in the titer of anti-BSA (gamma globulin) in the CSF of the rabbits with experimental allergic encephalitis, though the serum titers were comparable to those of the normal rabbits. Kabat and associates[13] have demonstrated that the concentration of gamma globulin is elevated in the CSF in experimental allergic encephalomyelitis. In the early stages, this increase may be associated with a general rise of protein in the CSF, although later the gamma globulin concentration may be disproportionately elevated. Vulpe and co-workers[14] showed that there is an increase of vascular permeability to radioactive labeled human serum albumin (RISA) in acute experimental allergic encephalomyelitis. Klatzo[15] has demonstrated a similar breakdown of the blood-brain barrier to fluorescein-labeled human serum albumin in this disease.

The data also show that bovine serum albumin, which is of lower molecular weight than gamma globulin, distributes itself in a similar fashion between the blood and the CSF. Our finding that BSA was not detected in CSF samples obtained at four hours differs from that of Fishman[16] who noted significant amounts of RISA in the CSF of the dog forty minutes after intravenous injection. This may be due to species differences or to the greater sensitivity of the radioactive methods. The average time required for equilibrium to be established between RISA in plasma and CSF was twenty hours, which is in agreement with our data.

BSA appeared to leave the CSF following intracisternal injection more rapidily than it entered it from the blood after intravenous administration. This result is in agreement with that of Courtice and Simmonds[17] who found that albumin tagged with Evans' blue dye attained a peak concentration in the plasma three to five hours after intracisternal injection. Dupont and associates[18] injected RISA intracisternally in dogs and noted a lag period of one to two hours prior to its appearance in the blood. In twenty-four hours, only 1 to 15 per cent of the radioactivity was left in the CSF. They interpreted this finding as an accelerated removal of excess protein from the subarachnoid space. Bowsher[19] has pointed out that the leptomeninx, the chief absorptive membrane of the CSF-containing system, is a part of the reticuloendothelial system. Whether this system is capable of producing antibody is at present unknown. However, rabbits which had received a single immunizing dose of BSA intracisternally produced circulating hemagglutinating antibody as early as the fifth day.[20] This finding is in agreement with that of Jankovic and co-workers[21] who noted circulating precipitins on the sixth day after injection of human gamma globulin into the lateral ventricle of rabbits by way of a cannula.

It thus appears that the CSF antibody titer reflects that of the serum in the normal animal. Relatively more antibody may be present in the CSF in inflammatory states, such as encephalomyelitis, which are characterized by an increase in the permeability of the blood-brain and blood-CSF barriers, thus allowing more serum proteins to enter the CSF.

SUMMARY

1. Antibodies were detected in the cerebrospinal fluid (CSF) of rabbits after either active or passive immunization with bovine serum albumin (BSA) or ovalbumin (OA). The ratio of the serum antibody titer to that of the CSF antibody titer varied between 100 and 1,600.

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The authors wish to ack
Miss Mary Roach and
Monique Fischer in carry

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2. Passive immunization of rabbits by the intravenous administration of either anti-OA or anti-BSA serum resulted in the appearance of antibody in the CSF within twelve hours. Peak CSF titers were attained within twenty-four hours.

3. The antibody titers in the CSF and blood subsequently declined in a similar fashion following passive intravenous immunization. This was a reflection of the half-life of the gamma globulin in the host.

4. Passive immunization of rabbits with acute experimental encephalomyelitis resulted in the appearance of a greater amount of antibody in the CSF as compared with passively immunized normal rabbits.

5. It was found that BSA could pass more rapidly from the CSF into the blood than in the reverse direction.

The authors wish to acknowledge the helpful advice of Miss Mary Roach and the technical assistance of Mrs. Monique Fischer in carrying out these studies.

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TAB B

to
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Eur. Neurol. *15:* 231–236 (1977)

The Transfer of IgG from Serum to CSF, Evaluated by Means of a Naturally Occurring Antibody

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Key Words. Antibodies · CSF · IgG · SSPE · MS · Blood brain-barrier

Abstract. Titres in CSF of natural IgG antibodies (Ab) to rabbit erythrocytes (RRBC) correlated well with titres in serum in normal individuals, indicating a passive transfer of the Ab. The ratio of CSF Ab to serum Ab mostly found was 1:400. The ratios of total IgG were slightly lower than the ratios of Ab to RRBC in normal individuals and in the majority of the diseases, except in multiple sclerosis and in subacute sclerosing panencephalitis (SSPE) where the ratios of total IgG were markedly elevated, indicating production of IgG in the central nervous system. In SSPE the ratio of measles Ab was even more elevated, suggesting that these antibodies contribute to the local IgG production. The ratios of Ab to RRBC in the two diseases were not elevated, or only slightly elevated. In normal and pathological CSF and in the Ab to RRBC all subclasses of IgG were found. In conclusion, the Ab to RRBC are considered convenient markers of serum IgG in the CSF.

Antibodies to rabbit erythrocytes (RRBC) are present in serum of most individuals throughout their lives (8). In a preceding paper (4) we reported the presence of IgG antibodies to RRBC in 94 % of 221 cerebrospinal fluids (CSF) from controls and patients with various diseases of the nervous system. Because of the simple method of detection we suggested the use of these antibodies as markers of antibody activity in the CSF, and they did prove useful for this purpose (3).

If the antibodies to RRBC are passively transferred from the blood, they may serve as markers for serum antibodies in the CSF. There is a need for such marker antibodies when examining the CSFs of patients with diseases where a local production of antibodies in the central nervous system may have taken place. In the present paper the correlation between serum and CSF with regard to titres of antibodies to RRBC and concentrations of IgG are reported.

[1] Fellow of the Norwegian Research Council for Science and the Humanities.

[2] The author thanks Mrs. *Turid Tynning* and Mrs. *Sigrid Mörner* for the valuable technical assistance.

Materials and Methods

Patients

The patients were divided into 9 groups on a clinical basis. The *control group* comprised patients with minor psychoneurotic disorders, hospitalized at the Department of Neurology with a tentative diagnosis of neurological disease, but whose complaints subsequently were found to be non-neurological. Their CSFs contained not more than 3 leucocytes/μl, and the total protein concentration, mean 33 mg/100 ml, was below 45 mg/100 ml. For the present purpose these patients were regarded as normal. The multiple sclerosis (MS) patients were selected according to *Allison and Millar*'s (1) probable MS. The mean total protein in the CSFs was 51 mg/100 ml. The patients with subacute sclerosing panencephalitis (SSPE) had typical histories and clinical pictures, and the titres of antibodies to measles virus in their CSFs were high. (Complement fixing antibodies were routinely determined at the Virus Laboratory, Department of Microbiology.) The mean total protein in the CSFs of these patients was 50 mg/100 ml. Patients with serous meningitis, acute encephalitis, subacute encephalomyelo-radiculitis and acute radiculoneuritis (Guillain-Barré) were included in a group designated *inflammatory diseases*. The mean total protein concentration in the CSFs was 195 mg/100 ml. Patients with clinical symptoms and roentgenologic signs of degenerative disease of the lumbar or cervical spine, with or without signs of disc prolapse, and also 6 patients with intraspinal tumour were included in a group designated *diseases of the spine*, subgroup A with CSF total protein below 50 mg/100 ml, the mean being 39, and subgroup B with CSF total protein 50 mg/100 ml or above, mean 93. Patients with head injuries contracted at least 1 year prior to the present examination and with evidence of brain lesions were included in the group *sequelae of head injuries*. The mean total protein in the CSFs was 37 mg/100 ml. The group *brain lesions of miscellaneous etiology* comprised patients with clinical or roentgenologic signs of brain lesions but the etiology was multiple or uncertain in the majority of the patients in this group. Some of them had a history of birth injury. The mean total protein in the CSFs was 48 mg/100 ml. Patients having a history of, and signs indicating, cerebral embolism, cerebral haemorrhagia, cerebral arteriosclerosis, or hypertensive encephalopathy, or sequelae following ruptured arterial aneurysm were included in the group *vascular brain diseases*. The mean total protein in the CSFs of this group was 41 mg/100 ml. One group comprised patients with *chronic degenerative and heredodegenerative diseases*. Diseases included in this group were amyotrophic lateral sclerosis, presenile dementia, idiopathic paralysis agitans, and the heredo-ataxias. The mean CSF total protein concentration was 62 mg/100 ml. Patients who did not belong to any of the groups mentioned were included in a group designated *other neurological diseases*. The mean total protein in the CSFs of these patients was 45 mg/100 ml.

Sera and CSFs

The sera and CSFs were collected on the same day and stored at −20 °C. Monovalent antisera to IgG and antiserum to human serum were the same as used earlier (4), and treatment with mercaptoethanol was performed as previously described (4). Monospecific antisera to subclasses of IgG were purchased from Nordic Immunological Laboratories, Tilburg, The Netherlands.

Titration of IgG Antibodies to RRBC

Indirect agglutination by CSF of RRBC with Coombs' test is mediated by IgG antibodies (4). The titration procedure described earlier (4) was performed with the CSFs, while sera were mercaptoethanol-treated prior to dilution. Recently we have modified the procedure, using microtitre equipment (Cook Engineering Company, Medical Research Division, Alexandria, Va.). In a plastic microtitration tray 100, 50, 25 and 25 μl of CSF were placed in the first, the second, the third, and the fourth well, respectively. The twofold dilutions started in the fourth well with a diluent consisting of .1 % inactivated normal rabbit serum in phosphate-buffered saline, pH 7.2 (PBS). The twofold dilution of serum started with a 1/100 dilution in the first well. 25 μl of a 0.5 % suspension of RRBC was added to each well, and after incubation at room temperature for 15 min the RRBC were washed twice in diluent. The washing procedure was performed by adding 100 μl of diluent to the resuspended RRBC in each well and then the trays were centrifuged at 200 *g* for 90 sec and the supernatant expelled. After washing, 25 μl of a 1/100 dilution of an antiserum to human IgG was added. This antiserum had the titre 8,000 in Coombs' test with anti-D-sensitized human Rh-positive erythrocytes. After incubation at room temperature for 15 min, followed by centrifugation at 200 *g* for 90 sec the placed in an almost vertical position for 1 agglutination was recorded using the patte sedimented erythrocytes at the bottom of unagglutinated erythrocytes drained dow drop like patterns, while the agglutinated e were adhering to the bottom of the well. T defined as the reciprocal of the highest dil agglutination

Estimation of IgG Content

Single radial diffusion in agar was pe described earlier (4). Appropriate dilutions of CSFs with high IgG concentrations wer and pairs of serum and CSF were tested wi agar antibody mixture.

Detection of Subclasses of IgG

Double diffusion in agar was performe to the instructions given by the antiseru tuer. Serial dilutions of specific antibodi or of concentrated CSFs were tested with normal human serum as control.

Elution of Antibodies from RRBC

To 100 ml of 1 % RRBC 1 g of hur Kabi, A/B Kabi, Stockholm) was added. A tion for 30 min at room temperature and the RRBC three times in PBS, the RR suspended to 3 ml and the antibodies we 56 °C. After 5 min of incubation at that the suspension was centrifuged for 5 mi and the supernatant was removed before time was lowered.

Statistics

The Wilcoxon test for two samples an man coefficient of rank correlation were u

Results

IgG Antibodies to RRBC

The titres in the sera varied fr 400. The geometric mean in t group was 800. The only disease gro significantly ($p < 0.01$) from the co

centrifugation at 200 *g* for 90 sec the trays were ...ced in an almost vertical position for 15 min. The ...utination was recorded using the patterns of the ...mented erythrocytes at the bottom of the wells. ...glutinated erythrocytes drained down in tear-...p like patterns, while the agglutinated erythrocytes ...e adhering to the bottom of the well. The titre was ...ined as the reciprocal of the highest dilution giving ...utination

Estimation of IgG Content

Single radial diffusion in agar was performed as ...ribed earlier (4). Appropriate dilutions of sera and ... CSFs with high IgG concentrations were prepared, ... pairs of serum and CSF were tested with the same ... antibody mixture.

Detection of Subclasses of IgG

Double diffusion in agar was performed according ... the instructions given by the antiserum manufac-...r. Serial dilutions of specific antibodies to RRBC ... concentrated CSFs were tested with a dilution of ...mal human serum as control.

Elution of Antibodies from RRBC

To 100 ml of 1 % RRBC 1 g of human IgG (γ-..., A/B Kabi, Stockholm) was added. After incuba-... for 30 min at room temperature and washing of ... RRBC three times in PBS, the RRBC were re-...ended to 3 ml and the antibodies were eluted at ...C. After 5 min of incubation at that temperature ... suspension was centrifuged for 5 min at 1,000 *g* ... the supernatant was removed before the tempera-... was lowered.

Statistics

The Wilcoxon test for two samples and the Spear-... coefficient of rank correlation were used.

Results

IgG Antibodies to RRBC

The titres in the sera varied from 100 to ...00. The geometric mean in the control ...p was 800. The only disease group differing ...ficantly ($p < 0.01$) from the control group was vascular brain diseases where the mean titre was 1,600.

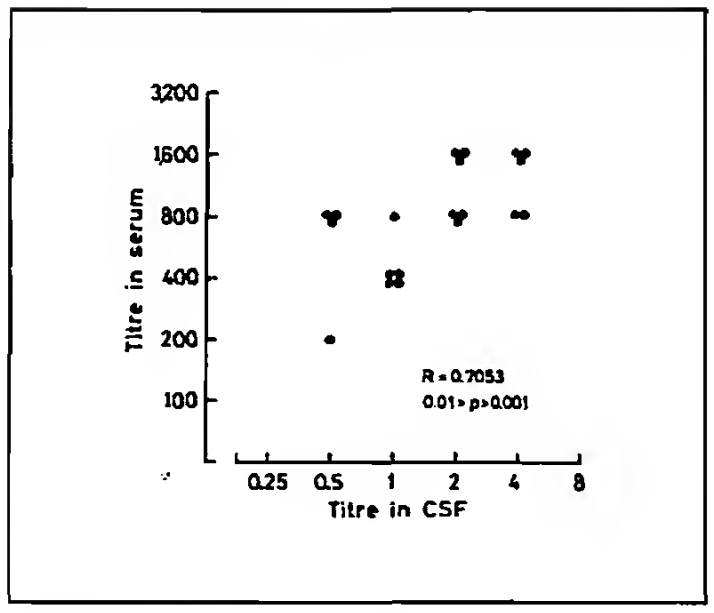

Fig. 1. Correlation between titres in serum and titres in CSF of antibodies to RRBC. Results obtained with specimens from individuals belonging to the control group. Each mark represents one individual.

To assure that IgM antibodies to RRBC did not overshadow the IgG antibodies detected with the antiglobulin test, all CSFs were tested for direct agglutination of RRBC. Only a small number of the CSFs agglutinated the RRBC directly, but each of them at titres much lower than those obtained with the antiglobulin test.

The correlation between titres of IgG antibodies to RRBC in serum and in CSF in the control group is shown in figure 1.

Ratio of CSF to Serum of IgG Antibodies and of IgG

The ratios of IgG antibodies to RRBC in the controls varied from 1:200 to 1:1,600, while the ratios in patients with diseases of the nervous system varied between 1:12.5 and 1:6,400 (table I). The most frequently occurring ratio was 1:400 in either group. Table II shows the ratios of IgG antibodies to RRBC and of IgG concentrations in the various groups

of patients. The ratios for antibody titres were significantly ($p < 0.01$) elevated in inflammatory diseases and in diseases of the spine with elevated CSF total protein. The ratios of IgG concentrations were significantly ($p < 0.01$) elevated in MS, in SSPE, in inflammatory diseases and in diseases of the spine with elevated CSF total protein. The ratios of CSF to

Table I. Distribution of number of individuals according to the ratio of CSF titre to serum titre of IgG antibodies to rabbit erythrocytes

Category of individuals	Ratio of CSF titre to serum titre									
	1:12.5	1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400
Controls (n = 20)					2	10	5	3		
Patients with diseases of the nervous system (n = 202)	1	3	5	19	42	82	37	9	3	1

Table II. Ratio CSF:serum of IgG antibodies (ab) to RRBC and of IgG

Patient group	Number in group	Ratio CSF:serum of			
		titre of ab to RRBC		concentration of IgG	
Control group	20	1:546[1]	(1:200–1:1,600)	1:645[1]	(1:244–1:1,088)
Multiple sclerosis	19	1:497	(1:200–1:1,600)	1:199	(1:58–1:436)
Subacute sclerosing panencephalitis	2	1:142	(1:100–1:200)	1:47	(1:37–1:56)
Inflammatory diseases	14	1:110	(1:12.5–1:400)	1:192	(1:9–1:894)
Diseases of the spine,					
Subgroup A[2]	16	1:475	(1:200–1:1,600)	1:689	(1:192–1:2,250)
Subgroup B[2]	42	1:214	(1:25–1:1,600)	1:374	(1:86–1:1,150)
Sequelae of head injuries	17	1:511	(1:200–1:3,200)	1:653	(1:194–1:2,250)
Brain lesions of miscellaneous etiology	24	1:476	(1:200–1:3,200)	1:645	(1:322–1:1,580)
Vascular brain diseases	13	1:551	(1:200–1:1,600)	1:715	(1:171–1:1,813)
Chronic degenerative and heredogenerative diseases	8	1:400	(1:200–1:800)	1:568	(1:138–1:971)
Other neurological diseases	47	1:440	(1:50–1:6,400)	1:579	(1:101–1:2,256)

Ranges in parentheses.

[1] Geometric mean ratio of titres, arithmetic mean ratio of IgG concentrations.

[2] In subgroup A CSF total protein concentrations are < 50 mg/100 ml, in subgroup B > 50 mg/100 ml.

Table III. CSF:serum ratios of antibo... IgG concentrations in two patients with...

SSPE patients	CSF:serum ratio of antibodies to	
	measles[1]	RRBC
No. 1	1:4	1:200
No. 2	1:8	1:100

[1] Complement-fixing antibodies.

...serum of complement binding a... measles virus, of IgG and of a... RRBC in the two patients with SSI... in table III.

Subclasses of IgG Present in the ... to RRBC and the CSFs

Double diffusion in agar of ...ntibodies eluted from RRBC at ... concentrated pool of normal CSF ...centrated CSF from one patient w... from one patient with MS respect... antisera specific for individual ... IgG, all showed the presence of ... classes.

Discussion

The positive correlation betw... serum and titres in CSF of antibo... in the control group, and the p... IgG subclasses in the antibodi... strongly indicate that the antibo... passively transferred from the ... CSF.

The geometric mean ratio of ... of antibodies to RRBC was in t...

were significantly ($p < 0.01$) IS, in SSPE, in inflammatory diseases of the spine with elevated protein. The ratios of CSF to

of CSF titre to serum titre of IgG

00	1:1,600	1:3,200	1:6,400
	3		
	9	3	1

concentration of IgG

1,600)	1:645[1]	(1:244–1:1,088)
1,600)	1:199	(1:58–1:436)
200)	1:47	(1:37–1:56)
:400)	1:192	(1:9–1:894)
1,600)	1:689	(1:192–1:2,250)
,600)	1:374	(1:86–1:1,150)
3,200)	1:653	(1:194–1:2,250)
3,200)	1:645	(1:322–1:1,580)
1,600)	1:715	(1:171–1:1,813)
800)	1:568	(1:138–1:971)
,400)	1:579	(1:101–1:2,256)

ions.
subgroup B ≥ 50 mg/100 ml.

Table III. CSF:serum ratios of antibody titres and of IgG concentrations in two patients with SSPE

SSPE patients	CSF:serum ratio of		
	antibodies to		IgG
	measles[1]	RRBC	
No. 1	1:4	1:200	1:56
No. 2	1:8	1:100	1:37

[1] Complement-fixing antibodies.

serum of complement binding antibodies to measles virus, of IgG and of antibodies to RRBC in the two patients with SSPE are shown in table III.

Subclasses of IgG Present in the Antibodies to RRBC and the CSFs

Double diffusion in agar of human IgG antibodies eluted from RRBC at 56 °C, of a concentrated pool of normal CSFs, of a concentrated CSF from one patient with SSPE and from one patient with MS respectively, against antisera specific for individual subclasses of IgG, all showed the presence of all 4 IgG subclasses.

Discussion

The positive correlation between titres in serum and titres in CSF of antibodies to RRBC in the control group, and the presence of all IgG subclasses in the antibodies to RRBC, strongly indicate that the antibodies are being passively transferred from the serum to the CSF.

The geometric mean ratio of CSF to serum of antibodies to RRBC was in the majority of the disease groups a little higher than the arithmetic mean ratio of IgG, but MS and SSPE contrasted markedly to this. In these two diseases the ratio of IgG was considerably higher than the ratio of antibodies to RRBC, indicating that local IgG production in the nervous system was taking place. In SSPE the ratio of measles virus antibodies was very high, but the ratio of RRBC antibodies normal or slightly elevated, indicating that there was neither increased production in the CSF of RRBC antibodies, nor cross-reaction between the increased measles-specific antibodies and RRBC. Consequently the antibodies to RRBC are suitable markers of serum IgG antibodies. Because the specific measles antibodies to a great extent contribute to the total IgG concentration in the CSF, the ratios of IgG in the SSPE patients were markedly elevated. The IgG ratio is therefore less suitable as parameter for evaluating the extent of transfer of IgG from serum to CSF.

The slightly lower CSF:serum ratio of IgG concentration than of IgG antibodies to RRBC in the majority of the diseases could lead to the suggestion that only selected subclasses of IgG are transferred to the CSF. All IgG subclasses were, however, found in selected CSFs, although the quantities remain to be investigated.

The ratio of CSF to serum of antibodies in the controls, 1:546, corresponds well with that presented by *Clarke et al.* (2) of antibodies to poliovirus, mean 1:505 and by *Sherwin et al.* (7) in experiments with rabbits, 1:400 to 1:800. *Norrby et al.* (6) in their control patients found a ratio of poliovirus-neutralizing enhancement antibodies of 1:440 and of adenovirus haemagglutination enhancement antibodies of 1:320.

In MS the ratio of serum to CSF of antibodies to RRBC, was not elevated. This is in accordance with the results reported by *Norrby*

et al. (6), who found no elevation of the ratio of antibodies to polio virus in 14 MS patients, and an insignificant elevation of the ratio of antibodies to adenovirus in 10 MS patients. On the other hand, *Clarke et al.* (2) found an elevated ratio of antibodies to polio virus in 19 MS patients, mean 1:236.

As markers for serum antibodies in the CSF, the natural occurring antibodies to RRBC have certain advantages: (1) they occur in practically all immunocompetent individuals above 3 months of age (5, 8); (2) they are detected in 94 % out of all CSFs (4), no other antibodies having been detected that frequently; (3) they are shown to be of the IgG class (4), and all 4 subclasses are represented; (4) cross-reactions with selectively increased antibodies in the CSF of MS and SSPE patients have not been found; (5) the detection method is simple, rapid, and less than 1 ml of unconcentrated CSF is needed.

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Eur. Neurol. *15:* 237–240 (1

Upper Lid Jerks

P. Salisachs and J. Lapres

Neurological Service, Hospit

Key Words. Lid jerks - l

Abstract. The authors r
(ophthalmoplegia, ataxia a
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TAB C

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Second Declaration
of Kenneth L. Rock, M.D.,
dated May 19, 2008

Eur. Neurol. *26:* 229–234 (1987)

0014–3022/87/0264–0229$2.75/0

Serum and Cerebrospinal Fluid Proteins and the Blood-Brain Barrier in Alzheimer's Disease and Multi-Infarct Dementia

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Department of Neurology, University of Helsinki, Finland

Key Words. Alzheimer's disease · Multi-infarct dementia · Blood-brain barrier · Intrathecal protein synthesis

Abstract. Serum concentrations of IgG, IgA, IgM, haptoglobin, transferrin, prealbumin and albumin quantitated nephelometrically in 22 patients with Alzheimer's disease (AD), 29 patients with multi-infarct dementia (MID) and their age-matched controls were normal. Cerebrospinal fluid (CSF) albumin and CSF/serum ratio for albumin were higher in AD and MID patients compared to controls, but no significant differences were found between AD and MID. Patients with MID had elevated CSF IgG, IgA, IgM and prealbumin levels compared to controls and to AD. An increased CSF IgG index was found in 5 MID patients but none of the AD patients. Thus, the blood-brain barrier permeability is often increased in MID as well as in AD. There is no increased intrathecal IgG synthesis in AD but it may occur in MID.

Introduction

Alzheimer's disease (AD) and multi-infarct dementia (MID) are the most common causes of dementia in the elderly.

The cause of AD is unknown, but virus-related [1], environmental [2] and amyloid-related [3] factors have been suggested to have a role in its pathogenesis. An altered blood-brain barrier (BBB) might serve as an entrance port into the brain tissue. The most common neuropathological lesions in AD are neurofibrillary tangles and senile (neuritic) plaques [4, 5]. In MID, multiple infarcts and arteriosclerotic changes, frequently associated with a damaged BBB, are scattered throughout the brain [6].

Investigations of humoral immunity in AD and MID have failed to reveal specific changes. However, elevated serum IgG and

Table I. The concentrations of different proteins in the serum and CSF of AD patients, MID patients and their age-matched controls

	Subjects		
	AD (22)	MID (29)	controls (22)
Serum			
IgG	12.5 ± 2.7	13.9 ± 3.8	13.4 ± 2.5
IgA	3.1 ± 1.5	3.2 ± 1.4	3.0 ± 1.5
IgM	1.4 ± 0.8	1.2 ± 0.6	1.4 ± 0.7
Haptoglobin	2.4 ± 0.9	2.4 ± 0.9	2.4 ± 0.9
Transferrin	2.9 ± 0.5	2.9 ± 0.6	2.8 ± 0.4
Prealbumin	0.25 ± 0.04	0.28 ± 0.08	0.25 ± 0.05
Albumin	41.9 ± 5.2	42.2 ± 5.9	43.0 ± 3.3
CSF			
IgG	34.3 ± 16.9[a,b]	46.8 ± 20.1[cc,c]	24.8 ± 8.9
IgA	4.2 ± 2.6[b]	6.3 ± 4.0[cc]	3.4 ± 2.0
IgM	0.9 ± 0.4[b]	1.2 ± 0.6[cc,c]	0.7 ± 0.2
Haptoglobin	1.8 ± 1.7	2.6 ± 3.0	1.8 ± 1.5
Transferrin	23.5 ± 5.8[a]	26.2 ± 9.0[cc]	19.5 ± 5.9
Prealbumin	18.0 ± 2.2[b]	19.5 ± 2.2[c,c]	16.0 ± 4.6
Albumin	204.7 ± 66.9[a]	232.0 ± 85.1[c,c]	156.2 ± 58.9

The values are in g/l for serum and mg/l for CSF (means ± SD). The number of patients in each group is given in parentheses.

[a] $p < 0.05$ for AD vs. controls.

[b] $p < 0.05$ for AD vs. MID.

[cc] $p < 0.01$, [c,c] $p < 0.001$ for MID vs. controls.

IgA in cognitively impaired persons [7] and elevated serum IgG in MID [8] have been reported.

A dysfunction of the BBB has been suggested in both AD [8, 9] and MID [8, 10–12], but no increase in the local synthesis of immunoglobulins within the central nervous system (CNS) in dementia has been demonstrated [8–13].

In order to determine possible alterations in BBB permeability and immune response of the CNS in common forms of dementia, we have investigated the serum and CSF proteins in patients with AD, MID and their controls.

Materials and Methods

Patients. We studied 22 ambulatory patients with AD, 29 patients with MID and 22 age-matched non-demented controls. Dementia was evaluated by a Luria-based neuropsychological test [14]. All patients

Table II. CSF/serum ratios (means ± SD) for various proteins in AD, MID and their controls

Proteins	Subjects		
	AD (22)	MID (29)	controls (22)
IgG	2.7 ± 1.4[aa]	3.4 ± 1.4[ccc]	1.9 ± 0.7
IgA	1.4 ± 0.7[b]	1.9 ± 0.7[ccc]	1.1 ± 0.4
IgM	0.8 ± 0.6	1.2 ± 0.9	0.8 ± 0.8
Haptoglobin	0.7 ± 0.6	1.0 ± 0.9	0.7 ± 0.4
Transferrin	8.2 ± 2.3[a]	9.1 ± 2.4[ccc]	6.9 ± 2.0
Prealbumin	73.8 ± 14.9	73.9 ± 22.6	65.2 ± 18.2
Albumin	5.0 ± 1.8[aa]	5.6 ± 2.1	3.6 ± 1.4

The number of paients in each group is given in parentheses.

[a] $p < 0.05$, [aa] $p < 0.01$ for AD vs. controls.

[b] $p < 0.05$ for AD vs. MID.

[ccc] $p < 0.001$ for MID vs. controls.

were severely or moderately demented. The clinical criteria for the diagnosis of AD and MID were as outlined by the American Psychiatric Association [15]. The diagnostic assessment included the patient's history, physical examination, laboratory tests, EEG and computed tomography (CT) of the brain.

There were 22 patients with AD (11 males, 11 females) with a mean age (± SD) of 67.2 ± 9.2 years (range 53–83 years). The MID series included 29 patients (14 males, 15 females) with a mean age of 68.2 ± 8.6 (48–86) years. Changes compatible with multiple brain infarcts were seen in all MID patients by CT imaging.

The control group consisted of 22 ambulatory, age-matched patients with a mean age of 67.4 ± 1.3 (57–80) years. They were patients examined because of neurological symptoms but they had no organic disease known to affect higher cortical functions.

Determination of the Proteins. IgG, IgA, IgM, haptoglobin, transferrin, prealbumin and albumin were determined nephelometrically with a Hyland Laser Nephelometer PDQ (Hyland Travenol, Costa Mesa, Calif., USA) [9].

Ratios and Indexes. The BBB function was determined by the concentration of CSF albumin and CSF/serum ratio of albumin [16]. The presence of intrathecal synthesis of an individual protein was determined by the following index [17]:

$$\frac{\text{protein (CSF)}}{\text{protein (serum)}} : \frac{\text{albumin (CSF)}}{\text{albumin (serum)}}$$

The two-tailed t test was used for the statistical comparison of the concentrations, ratios and indexes of the various proteins.

Results

Serum Proteins. The concentrations of various proteins in the serum of patients with AD, MID and their age-matched non-demented controls are given in table I. No significant differences between the groups were found.

CSF Proteins. The concentrations of IgG, transferrin and albumin were higher ($p < 0.05$) in AD as compared to controls, but the concentrations of IgG, IgA, IgM and prealbumin were lower ($p < 0.05$) in AD in comparison with MID (table I).

The levels of IgG, IgM, prealbumin and albumin ($p < 0.001$), as well as those of IgA and transferrin ($p < 0.01$) were significantly elevated in MID as compared to controls.

CSF/Serum Ratios. The ratios for IgG and albumin ($p < 0.01$) and transferrin ($p < 0.05$) were significantly elevated in AD in comparison with the controls (table II). The ratio for IgA was lower ($p < 0.05$) in AD than in MID.

In MID the ratios for IgG, IgA, transferrin and albumin were significantly elevated ($p < 0.001$).

CSF Indexes. The indexes for haptoglobin and transferrin were lower ($p < 0.05$) in AD than in controls (table III). The indexes for IgG and IgA were also lower ($p < 0.05$ and $p < 0.01$, respectively) in AD than in MID.

Table III. CSF indexes (means ± SD) for various proteins in AD, MID and their controls

Proteins	Subjects		
	AD (22)	MID (29	controls (22)
IgG	0.54±0.09[b]	0.61±0.10[cc]	0.53±0.08
IgA	0.28±0.08[bb]	0.34±0.07	0.33±0.09
IgM	0.17±0.13	0.23±0.16	0.24±0.36
Haptoglobin	0.13±0.07[a]	0.17±0.11	0.19±0.10
Transferrin	1.74±0.44[a]	1.74±0.46	2.04±0.51
Prealbumin	17.00±7.50	14.60±7.00[cc]	19.70±6.30

The number of patients in each group is given in parentheses.
[a] $p < 0.05$ for AD vs. controls.
[b] $p < 0.05$, [bb] $p < 0.01$ for AD vs. MID.
[cc] $p < 0.01$ for MID vs. controls.

The index for IgG was higher ($p < 0.01$), but that for prealbumin lower ($p < 0.01$) in MID than in controls. Five MID patients, but none of the AD patients, had an IgG index value higher than the mean control IgG index +2 SD.

Discussion

In the present study, no significant differences were found in the concentrations of serum proteins between AD, MID and their age-matched controls. Of the CSF proteins, only IgG, transferrin and albumin were increased in AD, while all proteins except haptoglobin were elevated in MID.

The elevation of CSF albumin and the CSF/serum ratio for albumin suggests an impaired BBB permeability in MID as well as in AD. The concept of increased permeability is further supported by the elevated concentrations of CSF IgG, IgA and transferrin combined with increased CSF/serum ratios for these proteins in both patient groups. Our results thus agree with those of Alafuzoff et al. [8] but are different from those of Leonardi et al. [10] who found BBB dysfunction only in MID. This disagreement may be due to differences in the selection and classification of the patients, because slightly different clinical criteria for AD and MID were used. Methodological differences, including the storage of the specimens, may also be a factor.

An increased permeability of the BBB has been demonstrated in MID both with immunohistochemistry [18] and quantitation of CSF and serum albumin (CSF-albumin, CSF/serum ratio for albumin) [8]. BBB changes have been suggested also in AD in a previous immunocytochemical study [19]. The BBB may also be altered in normal aged

persons [20]. A recent study demonstrated that the BBB was compromised equally in AD, mixed AD and MID, and in non-demented elderly subjects [21]. Our results showed, however, that BBB permeability is altered in a number of patients with common forms of dementia but not in the non-demented controls.

Because IgG and prealbumin were elevated in the CSF of MID patients while the CSF/serum ratios were normal, some consumption of these acute-phase proteins in their CNS might be possible. It has been suggested that prealbumin is associated with perivascular deposits in cerebral cortex in vascular dementia [18] and might serve as an indicator for such consumption phenomena.

The IgG index was increased in 5 of 29 MID patients, suggesting an increased intrathecal synthesis of IgG. Although the index has only a comparatively small and constant maximal error which results from the variation coefficients of the IgG and albumin assays, the influence of an increased BBB permeability cannot be excluded [22]. In AD there was no evidence for any increased intrathecal synthesis of IgG. The elevated CSF levels of IgG, IgA, IgM, transferrin, prealbumin and albumin in AD and MID are thus best explained on the basis of their diffusion from blood into the CSF through an impaired BBB.

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TAB D

to
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of Kenneth L. Rock, M.D.,
dated May 19, 2008

Antibody Guided Diagnosis and Therapy of Brain Gliomas using Radiolabeled Monoclonal Antibodies Against Epidermal Growth Factor Receptor and Placental Alkaline Phosphatase

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Twenty-seven patients with brain glioma were scanned using ^{123}I-labeled monoclonal antibodies against epidermal growth factor receptor (EGFR1) or placental alkaline phosphatase (H17E2). Successful localization was achieved in 18 out of 27 patients. Eleven out of 27 patients were also studied using a nonspecific control antibody (11.4.1) of the same immunoglobulin subclass and observable tumor localization was also achieved in five patients. The specificity of targeting was assessed by comparing images obtained with specific and nonspecific antibodies and by examining tumor and normal tissue biopsies after dual antibody administration. Ten patients with recurrent grade III or IV glioma who showed good localization of radiolabeled antibody were treated with 40–140 mCi of ^{131}I-labeled antibody delivered to the tumor area intravenously (n = 5) or by infusion into the internal carotid artery (n = 5). Six patients showed clinical improvement lasting from 6 mo to 3 yr. One patient continues in remission (3 yr after therapy), but the other five who responded initially relapsed 6–9 mo after therapy and died. No major toxicity was attributable to antibody-guided irradiation. Targeted irradiation by monoclonal antibody may be clinically useful and should be explored further in the treatment of brain gliomas resistant to conventional forms of treatment.

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Brain gliomas comprise ~60% of all primary CNS tumors and are challenging areas both for clinical oncologists and laboratory scientists (*1*). The prognosis of these tumors has not changed significantly in the last decade despite advances in surgery (*2*), radiotherapy (*3–5*) and chemotherapy (*6*). Postoperative irradiation may improve the quality of life and extend survival in many cases (*3*). It has been suggested that higher doses of radiation may contribute to increased survival but this should be tempered by the side effects that may arise from high doses of radiation (*4,5*).

Targeting of radiation using monoclonal antibodies is an attractive concept and encouraging responses have been described in some instances (*7*) including a case report of brain glioma (*8*). Intravenous administration of radiolabeled antibodies results in very low uptake by the tumor (*9*). It has been suggested (*8*) and shown in clinical (*10*) and preclinical (*11*) studies that the intra-arterial administration of radiolabeled antibodies may be advantageous in terms of improved tumor targeting. Selection of appropriate targets for monoclonal antibody guided therapy is a challenging area. We have selected epidermal growth factor receptor (*12–14*) as one of the targets. Epidermal growth factor (EGF) (*15*)

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promotes growth of a wide range of cell types, but in order to respond to EGF, a cell must express on its surface a receptor for EGF (*16*). It has been shown that a wide range of tumors (*17*) including some brain gliomas (*13,14*) express high levels of EGF receptors. Furthermore, in some cases, the tumor DNA shows rearrangement and amplification (*14*) compared to the EGF receptor gene normally present in human placental DNA. It is probable that a high concentration of EGF receptors facilitates growth of tumor cells in vivo (*18*) and it has been demonstrated that tumors with high levels of EGF receptors have a worse prognosis (*19*).

Our second target for antibody-guided localization and therapy was placental alkaline phosphatase (PLAP) (*20*). We previously found positive expression of PLAP on tumors (*21*) including some brain gliomas. The function of PLAP is not known at present, but it is possible that its presence on cells offers them a growth advantage. High levels of PLAP in some tumors have been associated with a bad prognosis (*22*).

Our study had three main objectives: (a) to investigate whether radiolabeled antibody can localize successfully in brain gliomas; (b) to test the specificity of localization by incorporating a nonspecific monoclonal antibody in imaging and biopsy studies; and (c) to assess toxicity and therapeutic efficacy of ^{131}I-labeled antibodies in the treatment of recurrent gliomas resistant to conventional therapy.

PATIENTS, MATERIALS, AND METHODS

Patients

Two groups of patients were studied. The first consisted of 27 patients, with known or suspected brain gliomas (Table 1 and 2) aged 14–71 yr (mean = 45 yr) were selected for the radioimmunolocalization study. Patients who later were found to have metastatic carcinoma were excluded from the study.

All patients were scanned by external scintigraphy immediately after the i.v. administration of the radiolabeled antibody, and then at 24 and 48 hr. Eleven patients were also imaged using a nonspecific antibody 2 wk after the study with the specific antibody. The studies with the specific and nonspecific antibody were performed in a similar fashion using the same isotope, the same route of administration and equal doses. The mean amount of injected protein per patient for external body scintigraphy was ~250 μg (200–300 μg).

Ten patients, aged 14–65 yr (mean = 41 yr), with recurrent grade III or IV glioma who previously showed good antibody localization, were treated with iodine-131- (^{131}I) labeled antibody delivered to the tumor area by infusion into the internal carotid artery (N = 5) or by intravenous administration (N = 5) (Table 2).

The second group consisted of seven patients. They underwent tumor resection and radioactivity in tumor tissue specimens was estimated, 3 days after injection of the EGFR1 and the nonspecific antibody labeled with ^{131}I and ^{125}I, respectively.

No patient had received chemotherapy or radiotherapy within 6 wk of the study. Written and informed consent was obtained prior to entry into the study. Prior to injection patients were skin tested for allergy to mouse immunoglobulin and were given 120 mg potassium iodide daily for 7 days for diagnostic, and for 28 days for therapeutic studies, starting one day before antibody administration.

Monoclonal Antibodies

EGFR1. This mouse IgG1 antibody binds to the native folded external domain of the human and rat EGF receptors (*23*). It does not react with the v-erb B protein probably because it recognizes sequences not present in the truncated molecule.

H17E2. This is a mouse IgG1 that was raised against purified plasma membranes of normal term placenta (*24*). It precipitates placental alkaline phosphatase activity at a single band of 67,000 D consistent with the molecular weight of PLAP (*20*). It also reacts with the leucine-inhibitable form of alkaline phosphatase found at low levels in the normal testis and is cross reactive with the placental enzyme. It does not react with other nonplacental forms of alkaline phosphatase (*24*).

11.4.1. This is a mouse IgG1 that was raised against the mouse H-2KK antigen (equivalent to human HLA antigen) and does not cross react with any human tissues (*25*).

Radiolabeling

Iodine-123 (AERE Harwell), ^{125}I (Amersham International IMS 30.1) or ^{131}I (Amersham International IBS30) was added to immunoglobulin (10 mg/ml) and the iodination procedure carried out in iodogen coated tubes (*26*). Iodination proceeded for 5 min at room temperature, and the radiolabeled IgG was separated from free radioiodine by gel filtration on Sephadex G50 using phosphate buffered saline, pH 7.4, as elution buffer (*9*).

Immunoreactivity

This was tested in an enzyme linked immunosorbent assay with solid phase antigen (*27*). Furthermore, comparison of antibody immunoreactivity before and after radiolabeling was tested in a direct radioimmunoassay, including competition with unlabeled antibody as previously described (*9*). Purity of the antibody preparations was assessed by FPLC (Pharmacia, Sweden).

Immunohistology

After counting, fresh frozen tissue sections of the tumors were tested in an indirect immunoperoxidase reaction for antibody reactivity. The concentration of the antibody used in immunohistochemistry was 10 μg/ml.

Kinetics

Blood samples were taken at various intervals and urine was collected for 5 days following the administration of the antibody either for imaging or therapy. Aliquots of the blood and urine were counted in a gamma counter along with standards of the injectate for clearance studies. The content of the whole blood was calculated by estimating the expected blood volume from the patients body surface area (*28*). Renal ^{131}I excretion was calculated and expressed as cumulative excretion. Protein bound iodine in the serum was quantitated by chromatography on a column with Sephadex G50.

Imaging Studies

All the patients were scanned immediately after antibody administration labeled with 10 mCi of ^{123}I and then at 24 and 48 hr. A large field-of-view camera (General Electric 400T gamma camera) was used with a low-energy collimator. In general, 200k counts were used to produce the early scans and 100k counts for later scans. All the patients after antibody treatment, when ^{131}I was decayed enough, were scanned using a high-energy collimator.

Biodistribution Studies

Tumor and normal brain tissues were removed at operation, 3 days after antibody administration. Samples were washed with PBS with heparin, blotted dry, weighed immediately on an analytic balance and counted in a gamma counter, in order to establish the percentage of injected dose per gram of tissue. Specificity index was defined as the percent of the injected dose per gram of specific antibody divided by the percent of the injected dose of the nonspecific antibody in tumor tissue. In addition to the specificity index, the tumor to normal brain ratio was assessed and defined as the percent of the injected dose per gram of administered antibody in the tumor divided by the percent of the injected dose in normal tissue. Biopsies from necrotic areas were excluded from the calculation.

Dosimetry

Macroscopic dosimetry calculations were performed on the evidence from biopsy and scans, as well as from body clearance data. No attempt at microdosimetry was made although it could be assumed that local variations in radioactive concentration would give rise to undefined errors in dose calculation. The basic formula for tissue dose from radiation distributed within that tissue is

$$K \times T\frac{1}{2} \times C = \text{absorbed dose (cGy)},$$

where K is a constant representing the absorbed fraction for a particular radioactive emmission and incorporates a time integration constant. The effective half-life in this tissue is $T\frac{1}{2}$ and C is the measured concentration in the tissue (*29*). The effective half-life is related to the physical and biologic half-lives by the following expression:

$$1/Tef = 1/Tb + 1/Tp.$$

Gamma camera images compared with images of known concentration of isotopes in suitable phantoms in the water bath provided the data to estimate the tumor uptake after both the scan and therapy injections (*30*). By using a similar geometric arrangement between patient and phantom studies direct comparison may be made and few corrections are needed to account for scatter and attenuation.

The bone marrow dose following the treatment was estimated by integrating the ^{131}I radioactivity in the blood over 200 hr after treatment, at which time most of the ^{131}I (>95%) had been excreted or decayed. Bone marrow is extremely vascular and macromolecules would rapidly reach equilibrium with the blood. Active marrow constitutes 2.2% of the body weight, that is 25 to 31% of the blood weight. It was assumed in our calculations that 25% of the integrated blood activity was in the bone marrow.

Therapy

Monoclonal antibodies radiolabeled with ^{131}I were delivered to the tumor area by a 5-min infusion into the internal carotid artery (N = 5) or by an i.v. administration (N = 5). Patients received i.v. antibody if their internal carotid artery could not

TABLE 1
Imaging Study with EGFR1 Monoclonal Antibody

Patient no.	Age/sex (yr)	Histology	Previous treatment	Specific AB	Nonspecific AB
1	14/F	Glioma (brain stem)	RT	(+)	
2	55/M	Glioma (brain stem)	RT	(+)	(+/−)
3	41/M	Glioma (grade III)	Surgery, RT	(+)	(−)
4	37/M	Glioma (grade III)	Surgery, RT	(+)	(−)
5	41/F	Glioma (grade IV)	Surgery, RT	(+)	
6	60/M	Glioma (grade IV)	RT	(+)	(+)
7	50/M	Glioma (grade IV)	Surgery, RT	(+)	
8	71/M	Astrocytoma (grade III)	Surgery, RT	(−)	
9	43/M	Glioma (grade III)	RT, chemotherapy	(+)	(−)
10	42/F	Astrocytoma (grade III)	Surgery, chemotherapy	(−)	
11	69/M	Astrocytoma (grade IV)	Surgery	(+)	(+)
12	66/M	Glioma (grade IV)	Surgery	(−)	

be satisfactorily or safely catheterized or if the blood supply of the tumor was not primarily derived from one internal carotid artery (e.g., the patient with brain stem glioma). We have not made a quantitative comparison between the arterial and i.v. routes of antibody administration in this small group of patients.

Response Evaluation

All patients underwent a pre-study evaluation consisting of history and physical examination as well as full blood count, biochemical profile, x-ray, and computed tomographic (CT) scanning. The response in each patient after antibody treatment was assessed clinically and radiologically. Clinical evaluation consisted of symptoms and signs resulting from increased intracranial pressure, seizure attacks, neurologic deficits with disturbances of motor, speech, sensory, visual, or intellectual function or personality changes. The patients who demonstrated an improvement in their clinical state (improvement in neurologic signs and Karnofski performance score) after antibody treatment were classified as having "clinical response". After antibody therapy there was no concomitant treatment added which could have improved the clinical state.

Computed tomograms were performed 4 wk after the treatment and then at various intervals, to compare the size of the tumor and the persistence or not of cerebral edema. Tumors that had decreased by 25% in two perpendicular diameters were classified as having a "radiographic response". In many cases it was not possible to obtain an accurate measure of tumor volume from a series of CT scans. This was largely because of the presence of edema, necrosis, and the varied shape of the tumor. The use of two perpendicular diameters in our measurements only provides a semiquantitative estimate of tumor volume change. Response categories were: (a) patients who clinically and radiologically were in response; (b) patients who clinically but not radiologically were in response; and (c) patients with no response.

Human Antimouse Immunoglobulin Response

Patients were seen weekly following treatment. Serum samples were obtained from all patients before and after antibody administration (10 days, 2 and 4 mo). The same murine monoclonal antibodies were used as antigen in the enzyme linked immunosorbent assay, as previously described (*31*).

RESULTS

Patients

Details of patients investigated are shown in Tables 1, 2, and 3. Details of patients treated are shown in Table 4. No reactions were recorded after the skin test with mouse immunoglobulin.

Radiolabeling—Immunoreactivity

Monoclonal antibodies were satisfactorily radiolabeled with labeling efficiency of ~90% and specific

TABLE 2

Imaging Study with H17E2 Monoclonal Antibodies

Patient no.	Age/sex (yr)	Histology	Previous treatment	Specific AB	Nonspecific AB
1	32/M	Astrocytoma (grade III)	Surgery, RT	(+)	(−)
2	63/F	Glioma (grade III–IV)	Surgery, RT	(+)	
3	41/M	Glioma (grade IV)	Surgery, RT, and chemotherapy	(+)	
4	26/M	Glioma (grade IV)	Surgery, RT	(+)	
5	65/F	Astrocytoma (grade IV)	Surgery, RT	(+)	
6	60/F	Astrocytoma (grade III)	Surgery	(+)	
7	19/M	Glioma (grade IV)	Surgery, RT, and chemotherapy	(−)	(−)
8	42/M	Astrocytoma (grade II–III)	Surgery, RT, and chemotherapy	(+)	(+)
9	34/M	Glioma (grade III–IV)	Surgery, RT, and chemotherapy	(−)	(−)
10	53/M	Astrocytoma (grade III)	Surgery, RT, and chemotherapy	(+)	
11	35/F	Glioma (grade II)	RT	(−)	
12	38/F	Glioma (grade II)	Surgery, RT	(−)	
13	52/M	Glioma (grade IV)	Surgery, RT	(+)	(+)
14	40/M	Glioma (grade IV)	RT	(−)	
15	36/F	Glioma (grade III)	Surgery, RT	(−)	

activity between 5–8 mCi/mg. No significant loss of immunoreactivity, or aggregate formation was found. In the presence of antigen excess more than 70% and 60% of iodinated EGFR1 and H17E2, respectively, was capable of binding to antigen.

Immunohistology

EGFR1 monoclonal antibody showed positive staining in an indirect immunoperoxidase reaction against the majority of glioma tissue sections. This was less clear with H17E2 antibody because only a small number of glioma tissues have been tested thus far (work in progress). We scored as positive when >30% of malignant cells within each glioma tissue reacted with antibody as visualized by low power microscopy.

Kinetics

Kinetic studies were performed with all antibodies used in this study. Blood clearance was not significantly different between ^{123}I- and ^{131}I-labeled antibodies or any one of the three antibodies used. Iodine clearance was biphasic with a mean half-life of the first component T½a = 20 ± 5.5 hr and of the second component T½b = 34 ± 8 hr. The cumulative urinary excretion of the ^{123}I and ^{131}I over 5 days was ~60% and 70% of the administered dose, respectively. The protein bound radioactivity in the serum using ^{123}I-labeled antibodies was 90% to 95% (mean 93%), and with ^{131}I-labeled antibodies for treatment was 85% to 96% (mean 91%).

Imaging Studies

Successful antibody guided localization of brain gliomas was shown in nine out of 12 patients who received EGFR1 radiolabeled monoclonal antibody and in nine out of 15 patients who received H17E2 radiolabeled monoclonal antibody. The specificity of targeting was studied in 11 patients by comparing imaging after administration of specific and nonspecific antibodies. Observable tumor localization was also achieved in five out of 11 patients who received the nonspecific monoclonal antibody. Figure 1 shows an antibody scan using EGFR1-^{123}I in a patient with glioma. Figure 2 shows an antibody scan of the head, 12 days after treatment.

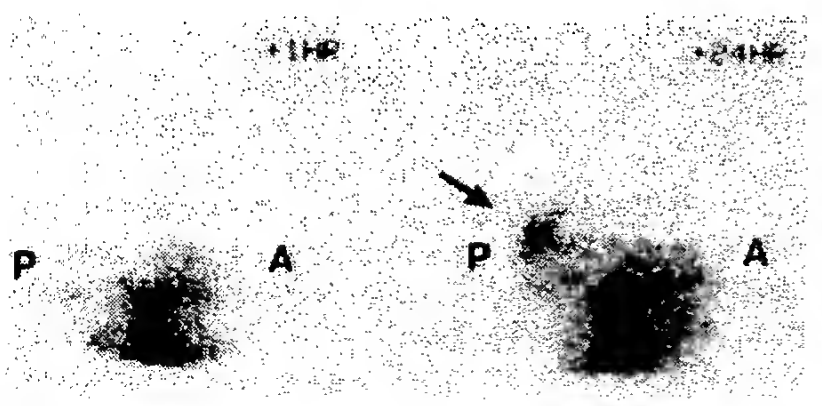

FIGURE 1
Antibody scan of the head with ^{123}I-labeled EGFR1 monoclonal antibody immediately after antibody administration and at 24 hr. The region of glioma is clearly seen (arrow). "A" and "P", anterior and posterior, respectively.

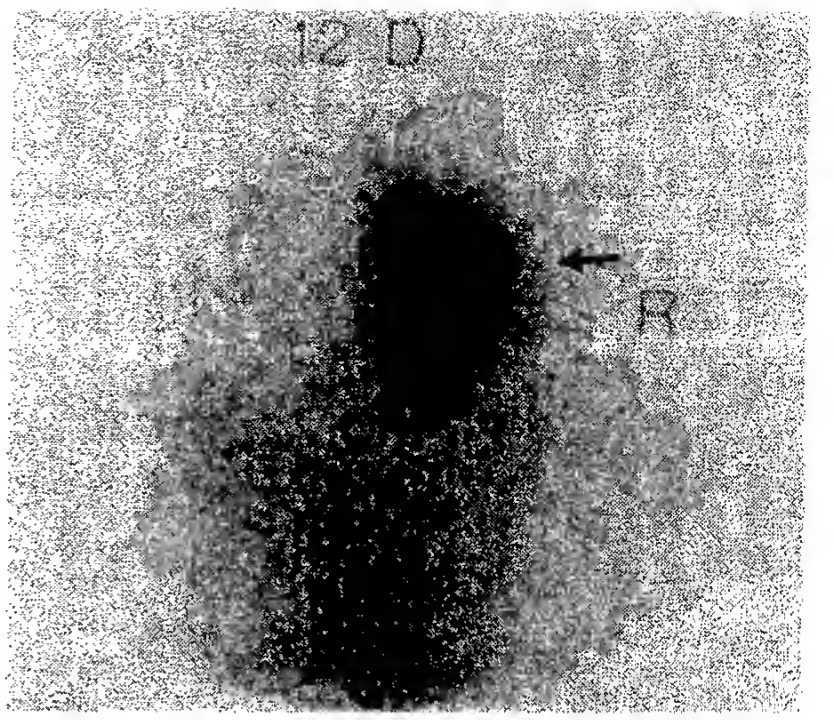

FIGURE 2
Antibody scan of the head 12 days after the treatment with ^{131}I-labeled H17E2 monoclonal antibody. The region of glioma is clearly seen (arrow).

Blodistribution Studies

An analysis of the percentage of injected dose per gram of tissue in the tumor specimens is shown in Table 3. The mean percentage of injected dose per gram of tumor and normal brain tissue with the specific antibody was ~0.004 and 0.0015, respectively. As shown in Table 3 more specific antibody accumulated in the tumor than nonspecific but the difference was small. The tumor to normal brain ratio with specific and nonspecific antibodies is shown in Figure 3.

Therapy Results

Ten relapsed patients, who had previously shown good localization of radiolabeled monoclonal antibodies were treated with 40–140 mCi of ^{131}I. The dose of ^{131}I administered was 40 mCi in two patients, 75 mCi in two patients, 100 mCi in four patients, 140 mCi in one patient and one patient was treated twice with 100 mCi each time. Six out of ten patients showed clinical improvement lasting from 6 mo to 3 yr. In these patients there was improvement in neurologic parameters, i.e., one patient showed movement in a hemiplegic arm and another patient partially regained her sense of balance without having further focal motor events. Neurologic improvement in these patients allowed for further reduction in their steroid dosages. In two of these six patients there was also radiologic improvement and one patient, who was treated twice continues in remission 3 yr after therapy. Four out of the six patients who responded initially, relapsed and died 6–9 mo after antibody therapy (Table 4).

TABLE 3
Biodistribution Studies

Patient no.	Age/sex (yr)	Histology (grade)	% ID/g of tumor Specific	Nonspecific	Specificity index (*)
1	28/M	Glioma (II)	0.0008 center 0.0009 edge	0.0033 center 0.0030 edge	0.24 0.30
2	42/F	Glioma (II)	0.0043 center	0.00018 center	23.89
3	40/F	Glioma (III)	0.0044 center 0.0020 edge	0.00326 center 0.0018 edge	1.35 1.11
4	56/M	Glioma (III)	0.0060 center 0.0030 edge	0.0032 center 0.0017 edge	1.87 1.76
5	48/F	Glioma (IV)	0.0050 center 0.0049 edge	0.00334 center 0.00196 edge	1.50 2.50
6	64/M	Glioma (IV)	0.0057 center	0.0030 center	1.90
7	32/M	Glioma (IV)	0.00566 center	0.00465 center	1.22

* Specificity index is defined as tumor ratio of specific versus nonspecific antibody.

Dosimetry

Static gamma camera images obtained at between 5 and 15 days after therapy showed a mean effective half-life in the tumor region of 40 hr. The maximum uptake by a 25-cc tumor was ~1 mCi from a 100-mCi injectate giving an upper limit for tumor dose of 1,250 cGy. The integrated dose is sharply dependent on the shape of the uptake/clearance curve representing the time before the first measurement at 4 days. These doses are only rough calculations because biopsies of treated areas were not obtained. Accurate dosimetry and microdosimetry in particular are very difficult to be performed clinically. Other studies of microdosimetry in experimental models showed a very wide range of tumor doses even within the same tumor mass (*32*).

The mean absorbed dose by bone marrow in the patients who received 100 mCi and 140 mCi was estimated to be ~140 cGy and 260 cGy, respectively.

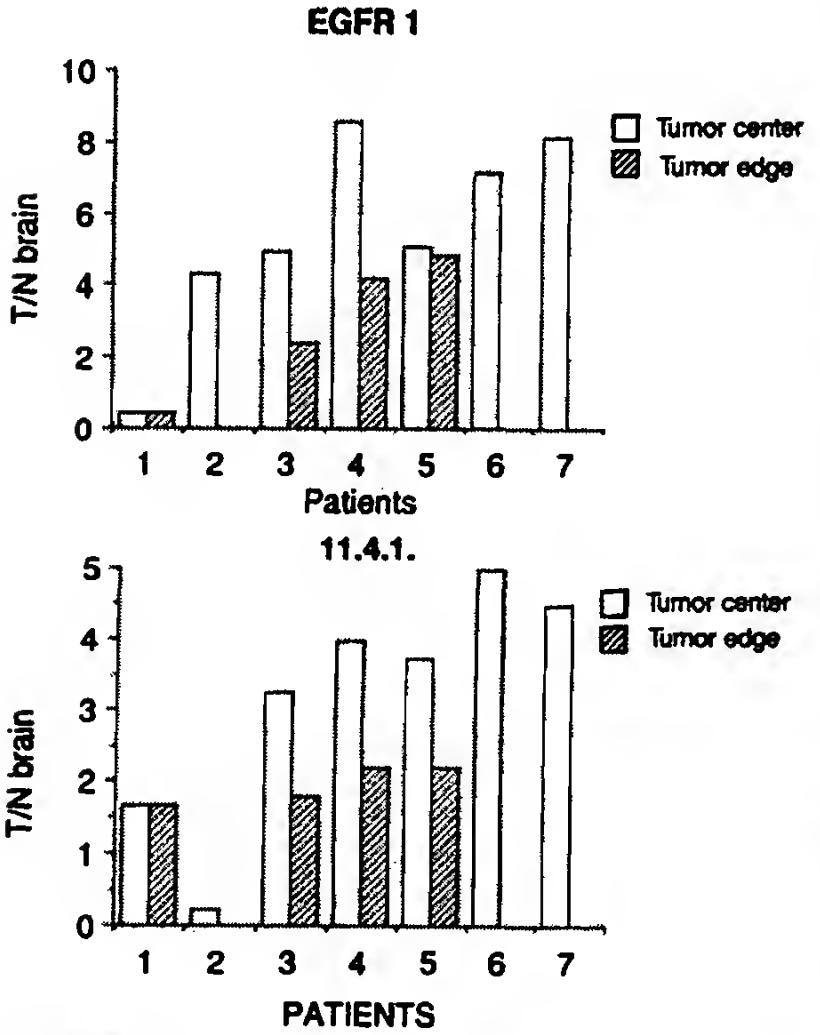

FIGURE 3
Tumor to normal brain ratio of antibody uptake 3 days after i.v. administration of EGFR1 monoclonal antibody (upper) and 11.4.1 monoclonal antibody (lower).

Toxicity

No acute toxicity was encountered in any of the patients. Furthermore, we did not observe any impairment in liver or renal function tests. The patients treated with 100 mCi developed mild neutropenia and thrombocytopenia (leucocytes 2–2.9 × 10^6 cells/l and platelets 50–74 × 10^9 cells/l) 3 to 5 wk after therapy. The patient who received 140 mCi developed moderate (leukocytes 1.0–1.9 × 10^6 cells/l and platelets 25–49 cells/l) but reversible thrombocytopenia (nadir at 30 days) and neutropenia (nadir 40 days) recovering 10–14 days after the nadir. The degree of marrow suppression was related to marrow doses: 140 cGy for mild toxicity; 260 cGy for moderate toxicity. Transient hemiparesis was observed in one patient (H17E2 3) due to the insertion of the intraarterial catheter into the internal carotid artery. This lasted for 5 min and was self limiting.

Human Anti-Mouse Response

One out of ten patients who had therapy developed human anti-mouse immunoglobulin response after treatment with radiolabeled monoclonal antibody. Nine patients did not develop an immune response above that demonstrated by healthy controls. Our ob-

TABLE 4
Therapy

Patient no.	Age/sex (yr)	Histology	Previous therapy	Antibody	Route	Dose	Clinical improvement	Radiologic improvement	Survival rate
EGFR1 #1	14/F	Glioma (brain stem)	RT	EGFR1	i.v.	40 mCi	Walking better Headache relieved	(–)	6 mo
EGFR1 #2	55/M	Glioma (brain stem)	RT	EGFR1	i.v.	40 mCi	No	(–)	42 days
H17E2 #3	41/M	Glioma (grade IV)	Surgery, RT, and chemotherapy	H17E2	i.a.	75 mCi	Headache relieved, no focal motor events	(–)	6 mo
H17E2 #5	65/F	Astrocytoma (grade IV)	Surgery, RT	H17E2	i.v.	75 mCi	Could walk unaided able to grasp objects with left hand	(–)	6 mo
H17E2 #4	26/M	Glioma (grade IV)	Surgery, RT	H17E2	i.a.	100 mCi	Became fully mobile	(+)	9 mo
H17E2 #2	63/F	Glioma (grade III–IV)	Surgery, RT	H17E2	i.a.	100 mCi	No	(–)	3 mo
EGFR1 #4	37/M	Glioma (grade III)	Surgery, RT	EGFR1	i.v.	100 mCi	Headache relieved Speech improved	(–)	6 mo alive
EGFR1 #5	41/F	Glioma (grade IV)	Surgery, RT	EGFR1	i.v.	100 mCi	No	(–)	4 mo
H17E2 #1	32/M	Astrocytoma (grade III)	Surgery, RT	H17E2	i.a.	140 mCi	No	(–)	4 mo
EGFR1 #11	41/M	Glioma (grade III)	Surgery, RT	EGFR1	i.a.	100 mCi (× 2)	Headache relieved, no seizures, walking improved	(+)	3 yr alive and in remission

servations on the phenomenon of human anti-mouse globulin response in patients has been described in detail elsewhere (*31*). In the patients studied by radioimmunoscintigraphy we could not detect human antimouse IgG antibody (other than preexisting response) during 4 mo of follow-up, even in those patients who received two administrations (specific and nonspecific antibody).

DISCUSSION

In a previous study (*8*), we showed that a radiolabeled tumor-associated monoclonal antibody, when given by an internal carotid artery infusion, could result in tumor regression and was of clinical benefit to a patient with recurrent grade IV glioma resistant to conventional therapy. This prompted us to perform this larger study to examine the reproducibility of that report, and to include controls such as the nonspecific antibody and different routes of administration (*33*) in order to assess if sufficient discrimination between antibody uptake in normal tissue and tumor could be achieved. This was primarily a study to determine toxicity and the limits of tolerance for normal organs. At the same time it provided limited information on efficacy of this approach. We carried out biopsies after administration of paired antibodies and we compared the uptake of both specific and nonspecific antibodies. As shown in Table 3 the absolute amount of specific antibody accumulated in the tumor was relatively small. However, in six out of seven cases, specific antibody (EGFR1) localized in higher amounts than nonspecific antibody (11.4.1). There are different factors which could account for the low accessibility of monoclonal antibodies in the brain tumors including the blood-brain barrier, lack of vascularity, tumor necrosis, etc. We have not made any measurements to assess the relation between the disruption of the blood-brain "barrier" and the antibody uptake in these patients. However, it is likely that breakdown of the blood-brain "barrier" in the tumors is, at least, in part responsible for both the nonspecific and the specific antibody uptake. Our findings from both the biopsy as well as imaging data demonstrate an element of nonspecific uptake. In five out of nine patients we showed positive radioimmunolocalization

of tumor with the nonspecific antibody. The relation between specific and nonspecific tumor immunolocalization is a complex phenomenon which may be different for every antibody-tumor system. This problem has not been adequately highlighted in the past by performing dual antibody radioimmunoscintigraphy studies. Therefore, for meaningful antibody-guided imaging studies of brain glioma we recommend the use of specific and nonspecific antibodies so that the issue of specificity can always be resolved.

There is a wide choice of antigens for targeted radiotherapy using monoclonal antibodies. There have been several reports of monoclonal antibodies reacting against human gliomas (*34–38*). We selected two molecules which, in addition to their increased expression on brain gliomas, may play a fundamental role in carcinogenesis and tumor promotion. Previous in vitro studies have shown a relationship between EGF receptor concentration and tumor growth (*18*). Gliomas express epidermal growth factor receptors (*13,14*) and this expression may play a role in carcinogenesis (*39, 40*). Comparison of the complete sequence of the EGF receptor gene with that of a transforming protein (V-erb B) present in avian erythroblastosis virus, showed that the latter was homologous with the transmembrane and cytoplasmic domains of the EGF receptor, but lacked the majority of extracellular sequences (*41*). One of the monoclonal antibodies used in this study (EGFR1) (*23*) binds to the external domain of the human EGF receptor but does not react with the V-erb-B protein, presumably because it does not recognize sequences present in the truncated molecule. Prior immunohistochemical testing of brain gliomas produced positive staining with EGFR1 antibody, indicating that most of these tumors synthesize the complete molecule of EGF receptors. Placental alkaline phosphatase (PLAP) may be another suitable target (*20*). It is normally found on term placenta, but its function is not known at present. Its ectopic expression on rapidly dividing tumors, such as germ cell neoplasms of the testes, may indicate that this enzyme is associated with rapidly progressing tumors. In ovarian cancer, high levels of serum PLAP are associated with a poor prognosis (*22*). The fact that PLAP is expressed at low levels or not at all in normal tissues, allows a further advantage with regard to tumor targeting, in contrast to EGF receptors that are present at high levels in some normal tissues such as the small bowel. However, it is not clear, what proportion of human gliomas express PLAP (work in progress).

The methods calculating radiation doses to tissues after the injection of a radioactively labeled compound are well documented (*29*). By using the Medical Internal Radiation Dosimetry Committee formula a dose can be ascribed to each individual organ and to the whole body provided a knowledge of the physiologic pathways and kinetics has been obtained. The specific information required is the amount of radioactivity, the residence time in any organ, and the size of that organ. In the case of the patients in this study, these three sets of data were obtained from separate groups of patients since those who were selected for treatment were not those who underwent the biopsy procedure. The size of the tumor was assessed where possible by CT scan. The radioactive concentration in the tumor was measured by either biopsy at 72 hr or by gamma camera images following the scanning injection, or between 4–15 days after the treatment when the activity had fallen to a manageable level, and by comparing these to images of calibrated water phantoms. The residence time in the tumor was also calculated from successive gamma camera images over a period of days. Each measurement has its own peculiar limitation and associated error. The most pessimistic dose estimates use uptake data from the biopsy and residence time from the diagnostic scans and assume a uniform infusion of radioactivity throughout the diseased tissue. Using this straightforward method the resulting tumor doses are in the region of only 100 or 200 cGys. This value could scarcely have had any beneficial effect. There is, however, some encouraging evidence to suggest that an effective adjuvant dose had been delivered to the tumor. First, the treatments were delivered, where possible, by way of the carotid artery, offering an immediate advantage of perhaps a factor of two (*10,11,33*). Second, the percentage of injected radioactivity in the tumor region at 4–15 days after treatment appeared to be more than that at the same time after the diagnostic scans indicating a value of 0.02% of the injected dose per gram of tumor. Third, the time of residence in the tumor following the treatment dose again would indicate a higher initial uptake although early local concentrations could not be measured because of the high background activity. The calculated doses are not sufficient on their own to sterilize brain gliomas. On the other hand, such doses could make an important contribution if used in conjunction with radical external beam radiotherapy in patients with poor prognosis grade III or IV gliomas.

Bone marrow toxicity was noted at doses of 100 mCi of ^{131}I-labeled antibody which was correlated with the estimated bone marrow dose. The hemopoietic bone marrow is extremely vascular and is irradiated by its circulating blood. We have estimated the radiation dose to marrow to be 140 cGy in patients receiving 100 mCi and 260 cGy in patients receiving 140 mCi of ^{131}I. These findings are in agreement with our previous studies where ^{131}I-labeled monoclonal antibodies were administered for the treatment of advanced ovarian cancer (*7,42*).

We were encouraged that six out of the ten patients treated showed clinical responses even after only one therapeutic administration. One patient, who was

treated on two occasions, continues in remission 3 yr post-therapy. We are not certain that the theoretic advantages of intraarterial infusion of drugs in terms of increased drug delivery to the tumor and decreased systemic toxicity (*33*), are applicable to macromolecules such as antibodies. The so-called first-pass advantage should be minimal in the case of macromolecules such as immunoglobulins, if the vascular and tumor areas were considered as a two-compartment model. Previous clinical (*10*) and recent preclinical studies (*11*) have shown an advantage for the intracarotid administration of antibodies. A possible explanation for this may be that intraarterial administered antibody sequestrates into a tumor "third space" which may act as a reservoir allowing for slow release and access to tumor antigen (*11*). We recognize that there is a case for the study of mathematic modeling in relation to arterial and venous methods of administration. We did not measure first-pass uptake at the time of treatment because of the difficulties of using a gamma camera in the angiography/catheterization room.

On the basis of this data further studies should be conducted to determine the exact role of the monoclonal antibodies for the treatment of brain gliomas. These results may be improved if one could use smaller molecules such as antibody fragments or genetically engineered fragments (*43*), radionuclides such as yttrium-90 (*44*) that may have more favorable radiobiologic characteristics than ^{131}I as well as drugs such as mannitol which utilize osmotic blood-brain barrier disruption and could increase antibody uptake (*45*).

In conclusion, we have shown that monoclonal antibodies against EGF receptors and placental alkaline phosphatase can be used to target brain gliomas but the difference between specific and nonspecific antibodies was small. In fact 50% of our patients with positive specific antibody scan showed successful immunolocalization with nonspecific antibody. In six out of ten patients with recurrent glioma who underwent antibody-guided therapy we demonstrated a clinical benefit, with no significant toxicity. Monoclonal antibody guided irradiation may be clinically useful and should be investigated further in the treatment of brain gliomas resistant to conventional forms of treatment.

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TAB E

to
Second Declaration
of Kenneth L. Rock, M.D.,
dated May 19, 2008

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A Saturable Mechanism for Transport of Immunoglobulin G across the Blood–Brain Barrier of the Guinea Pig

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The existence of an immunological blood–brain barrier to homologous blood-borne immunoglobulin G (IgG) was investigated in the guinea pig using a vascular brain perfusion technique *in situ*. Cerebrovascular unidirectional transfer constants (K_{in}) for ^{125}I-labeled IgG (2.5 μg/ml) estimated from the multiple-time brain uptake data, ranged from 0.53 to 0.58 ml min^{-1} g^{-1} × 10^3 in the parietal cortex, hippocampus, and caudate nucleus, the transfer rate being some 10 times higher than that for [^{3}H]dextran (MW 70,000). In the presence of 4 mg/ml unlabeled IgG, unidirectional blood to brain transfer of ^{125}I-IgG was markedly inhibited. Immunohistochemical analysis of the brain tissue after vascular perfusion with unlabeled IgG revealed a distribution of the blood-borne immunoglobulin in the endothelial cells of microvessels and in the surrounding perivascular tissue. It is concluded that there is a specific transfer mechanism for IgG at the blood-brain barrier in the guinea pig, which is saturated at physiological plasma levels of IgG.

INTRODUCTION

Central nervous system (CNS) is generally regarded as an immunologically privileged site, a concept based on the pioneering work of Medawar (15) who showed that incompatible heterologous tissue grafts may be accepted by central nervous tissue. However, more recent work has shown that most of the effector cells involved in the immune response are present, in low numbers, within the CNS (1), and moreover, the blood–brain barrier (BBB) may permit some immunocompetent cells to penetrate the brain (16). On the other hand, the possibility that a humoral response elicited outside the CNS may affect the brain via circulating antibodies has been rejected because of the presumed impermeability of the blood–brain and blood–CSF barriers to large water soluble molecules, such as proteins and peptides. It has been suggested that their blood–brain exchanges may occur only in high permeability regions, such as the circumventricular organs and choroid plexus (12) unless special mechanisms are present at the BBB (3). This concept has been supported by electron microscopy studies with the plant enzyme, horseradish peroxidase (HRP), the entry into the brain across capillaries being restricted by the presence of a continuous layer of endothelial cells closely connected by tight junctions, and by little capacity for vesicular transport (21, 4). On the other hand, it has been demonstrated that high vascular doses of HRP may penetrate into the brain across the segments of some cerebral arterioles (27).

However, considerations that apply to blood–brain transport of HRP and micro-HRP do not necessarily apply to the BBB transport of other studied proteins and peptides. For example recent *in vitro* work has revealed that a number of biologically important proteins and large peptides may bind to capillary endothelial cells; this is followed by subsequent internalization and exocytosis of the engulfed molecules, giving rise to the concept of receptor- and/or carrier-mediated transcytosis (17, 18). The presence of the specific blood-to-brain transport systems for a variety of brain peptides has been also shown at the BBB under *in vivo* experimental conditions (33).

Since there has been an apparent lack of direct experimental evidence respecting BBB permeability to immune proteins (reviewed in (5)), we have employed a recently developed vascular brain perfusion method (29) to measure transport of blood-borne homologous IgG across the BBB of the guinea pig *in vivo*.

METHODS

Vascular Perfusion of the Guinea Pig Forebrain

Adult guinea pigs of either sex weighing 250–300 g were anesthetized with 30–35 mg/kg thiopentone sodium. Perfusion of the guinea pig ipsilateral forebrain was performed through the right common carotid artery which was connected to an extracorporeal perfusion circuit as previously described in detail by Zlokovic *et al.* (29). The perfusion medium consisted of 20% sheep red cells (oxygen carrier) and artificial plasma salts (composition as previously reported (29)) with dextran (MW 70,000; 48 g liter^{-1}). Just before the perfusion started the

0014-4886/90 $3.00

contralateral carotid artery was ligated and immediately after the start of perfusion the jugular veins were cut to allow drainage of the perfusate. Perfusion pressure (13 to 16 kPa) and pCO_2 (5–5.5 kPa) were standardized to maintain cerebral blood flow in the ipsilateral forebrain at about 1 ml min^{-1} g^{-1} brain tissue. In all experiments the effective perfusion pressure was maintained 1–3 kPa above the animals' own arterial blood pressure to ensure the functional separation between the artificial and vertebral circulations as previously described in detail (29, 33). The values of effective perfusion pressure were well below the threshold for opening the barrier as described by others (2, 24). During guinea pig vascular brain perfusion, the threshold for hypertensive opening of the BBB was between 21 and 26 kPa at pCO_2 of 5 to 5.5 kPa (29). Under present perfusion conditions, it has been shown that the metabolic state of the brain, as indicated by water content, electrolyte ratios, ATP, ECP, and lactic acid contents, remains apparently normal, suggesting no ischemic changes in the hippocampus, parietal cortex, and caudate nucleus during brain perfusion (29).

Isotopically ^{125}I-labeled immunoglobulin G (IgG) solutions at concentrations of 2.5 μg/ml, in the absence and presence of unlabeled IgG, were introduced into the perfusion circuit by a slow-drive syringe at a known rate and concentration. At times, ranging from 1 to 20 min, the perfusion was terminated by cutting the right common carotid artery and decapitation of the animal. The brain was removed and dissected into different regions before preparation for scintillation counting. In a separate series of experiments brains were perfused with unlabeled homologous IgG for 10 min, followed by preparation for immunocytochemistry.

Scintillation Counting

The ipsilateral forebrain was divided into the following regions: parietal cortex, caudate nucleus, and hippocampus. Brain samples of approximately 100 mg wet wt were dispensed into preweighed scintillation vial inserts and plasma samples of 25 μl (supernatant of centrifuged perfusion medium) were taken for counting. For gamma counting the samples and standards were counted directly on an LKB gamma spectrometer. In a separate series of experiments when [^{3}H]dextran was perfused in the absence of radiolabeled ^{125}I-IgG, samples were solubilized overnight in 0.5 ml of soluene (Packard), and before counting they were treated with 4 ml of scintillation fluid (0.125 g of 1,4-di-2(5-plenyloxazolyl) benzene (POPOP) and 15 g of 2,5-diphenyloxazole (PPO) per 2.5 l of toluene). Samples for beta counting were measured on an LKB Spectral beta scintillation spectrometer at a low energy channel using an internal quench curve program.

Isotopically Labeled Substances

IgG was labeled with ^{125}I by the micro-chloramine-T method (11) modified by adding a 10-fold excess of chloramine-T as well as phosphate-buffered saline (PBS) of *p*H 7.4. Unbound iodide was removed by gel filtration on Sephadex G-200 and homogeneity was verified by thin-layer chromatography. The specific activity was about 0.1 μCi/μg; this enabled the use of highly radioactive perfusion fluids at concentrations about three orders of magnitude lower than normally found in guinea pig's own blood (34). [^{3}H]Dextran (MW 70,000) was purchased from Amersham.

Unlabeled Substances

Guinea pig IgG was obtained from Sigma.

Calculation of the Unidirectional Blood-to-Brain Transfer Constant, K_{in}

The unidirectional blood-to-brain transfer constant, K_{in}, for ^{125}I-IgG in the presence and absence of different concentrations of unlabeled IgG was estimated from the multiple-time brain uptake data, during the first 20 min of perfusion, as previously reported (29, 33). The following equation was employed,

$$C_{BR}(T)/C_{PL}(T) = K_{in}T + V_i,$$

where $C_{BR}(T)$ is the radioactivity of IgG measured per unit mass of brain at Time T, and $C_{PL}(T)$ is the constant radioactivity of immunoglobulins per unit mass of plasma during the perfusion; T is the time when perfusion is terminated. The radioactivity of IgG in the arterial inflow (C_{PL}) is constant under present experimental conditions. The equation defines a straight line with a slope, K_{in}, and an ordinate intercept, V_i, which includes the possibility of initial distribution of the solute in a rapidly reversible compartment(s).

Immunohistochemical Studies

Brains were vascularly perfused with either 4 mg/ml unlabeled IgG or only with a perfusion medium, for a period of 10 min, followed by perfusion with a fixative 2% PLP (Periodate–lysine monochloride–paraformaldehyde) for the next 15 min. The brain was then removed and prepared for immunohistochemistry. The avidin–biotin–peroxidase method as previously described (34) involved incubations with (a) anti-guinea pig IgG (raised in goat), (b) biotinylated anti-goat IgG, (c) avidin–peroxidase, and (d) diamino benzidine (specific substrate for peroxidase reaction). Preparations were lightly counterstained with hematoxylin–eosin.

RESULTS

Figure 1 represents the ipsilateral parietal cortical uptake, as a function of time, of carrier-free ^{125}I-IgG (n = 10) and of ^{125}I-IgG in the presence of 0.06 mg/ml unla-

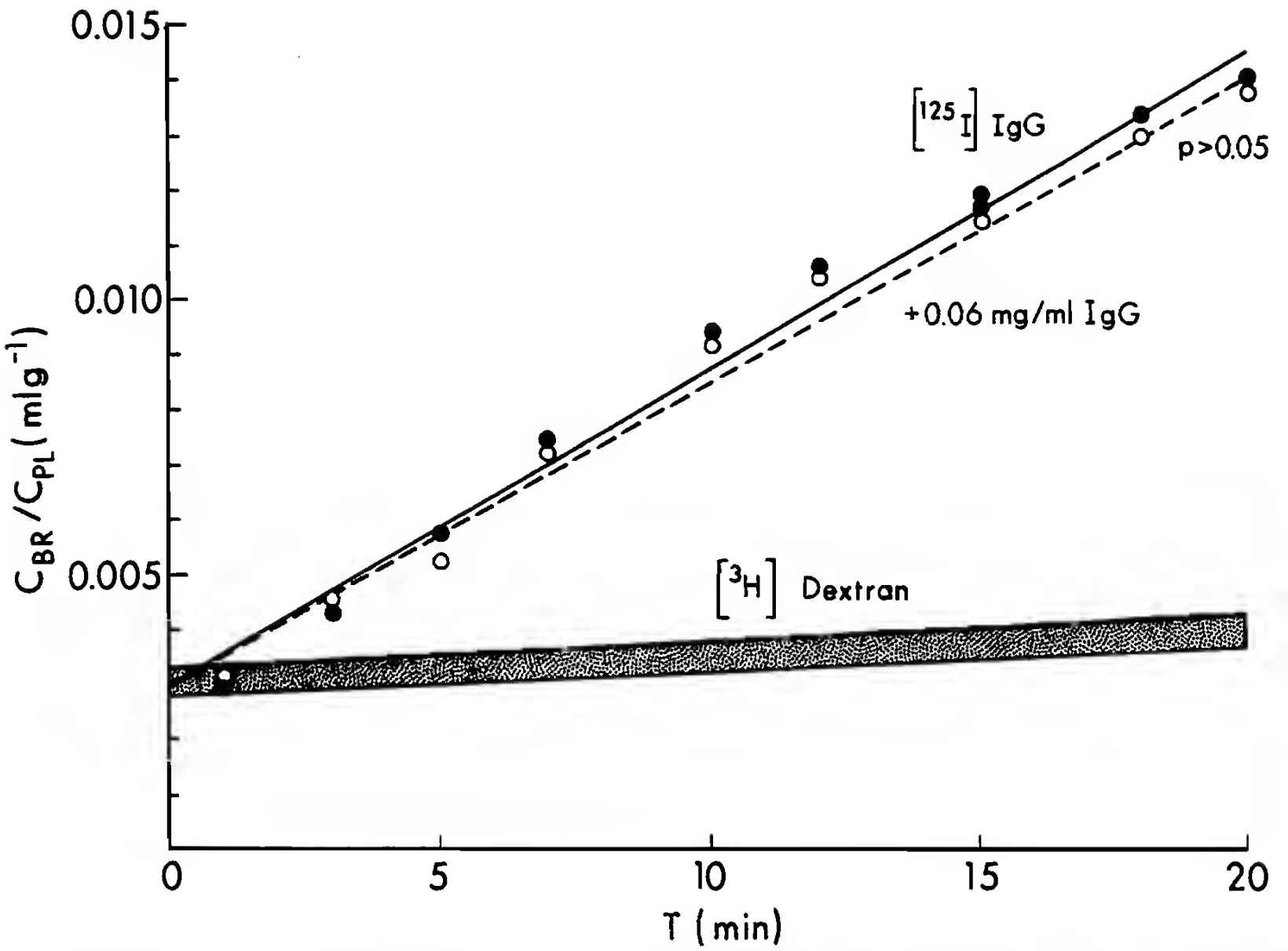

FIG. 1. Kinetics of entry of homologous ^{125}I-IgG (2.5 μg/ml) into the parietal cortex of the perfused guinea pig brain in the absence (solid points and line) and presence of 0.06 mg/ml unlabeled IgG (open points and interrupted line). ^{125}I radioactivity, cpm g^{-1} brain/cpm ml^{-1} plasma perfusate, is plotted against perfusion time, T. The shadowed area represents [^{3}H]dextran space determined in a separate series of experiments. Each point represents a single experiment; slopes of the regression lines were compared by analysis of variance (P).

beled IgG ($n = 9$) and, in a separate series of nine brains, the uptake of [^{3}H]dextran (MW 70,000), expressed as the ratio of brain and plasma activities, C_{BR}/C_{PL}, plotted as a function of time of perfusion. It can be seen that the dextran uptake, which is illustrated as a shadowed area defined by dextran $K_{in} \pm$ SEM and $V_i \pm$ SEM, stays virtually unchanged during the time of perfusion, thus representing the rapid filling of the vascular space by the tracer under experimental conditions. ^{125}I-IgG exhibits a moderate degree of penetration into the parietal cortex giving a C_{BR}/C_{PL} value of 0.003 at 1 min and rising to 0.014 at 20 min. When ^{125}I-IgG was perfused together with unlabeled IgG (0.06 mg/ml) C_{BR}/C_{PL} values were only a little reduced at some perfusion times which was not statistically significant. Figure 2 shows time-dependent ipsilateral hippocampal uptake of ^{125}I-IgG in the absence and presence of unlabeled IgG at concentrations as high as 4 mg/ml. Significant differences in the slopes of the regression lines ($P < 0.01$) were obtained under the different experimental conditions, indicating a marked self-inhibition effect which can be seen at all perfusion times. K_{in} and V_i values for ^{125}I-IgG calculated from the slopes and intercepts of the regression lines are shown in Tables 1 and 2. From Table 1 it can be seen that the values for K_{in} did not vary significantly from one region of the brain to another. Penetration of ^{125}I-IgG was about an order of magnitude higher than that for the inert polar dextran molecule (MW 70,000). There was no significant difference between the values of V_i for the two molecules, indicating that they both rapidly occupied the initial equilibriating space. Table 2 shows that low concentrations of unlabeled IgG (0.06 mg/ml) did not significantly affect the kinetics of entry of ^{125}I-IgG, whereas a marked self-inhibition was obtained in the presence of a higher concentration (4 mg/ml), the transfer-constant being reduced by 76 to 79%. If the transfer-constant for dextran is used as a background level, reflecting mainly the filling of the vascular space, the self-inhibitory effect produced by 4 mg/ml of IgG ranges between 86 and 87%. Immunohistochemical analysis of the brain tissue following perfusion with unlabeled IgG (4 mg/ml) confirms the kinetic studies in so far as it demonstrates penetration of IgG into the brain. Thus Figs. 3A and 3B illustrate immunohistochemical reaction of parietal cortex and hippocampus perfused for 10 min with the medium only (control) and with IgG, re-

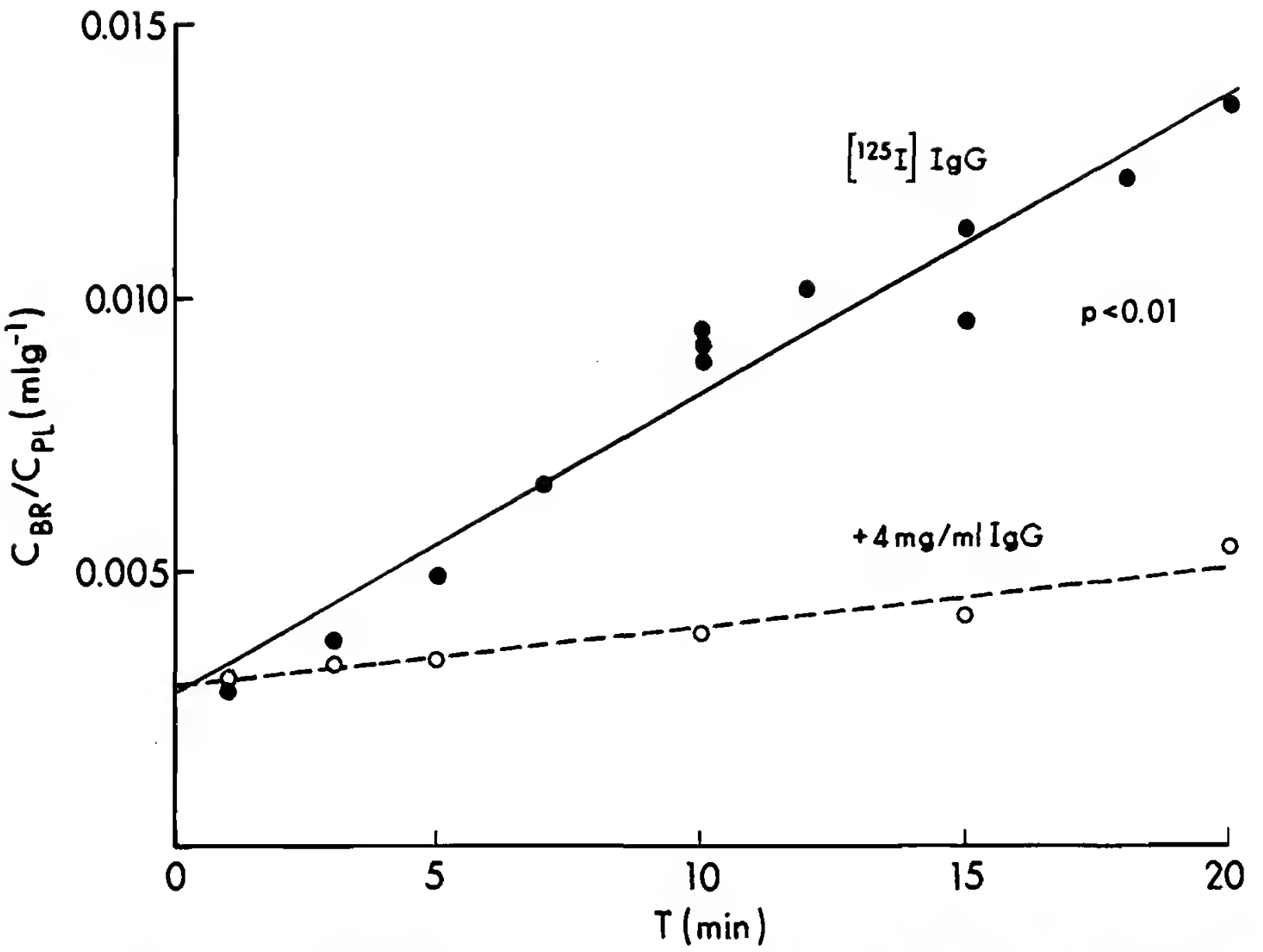

FIG. 2. Kinetics of entry of homologous [^{125}I-IgG (2.5 μg/ml) into the hippocampus of the perfused guinea pig brain in the absence (solid points and line) and presence of 4 mg/ml unlabeled IgG (open points and interrupted line). ^{125}I radioactivity, cpm g^{-1} brain/cpm ml^{-1} plasma perfusate, is plotted against the perfusion time, T. Each point represents a single experiment; slopes of the regression lines were compared by analysis of variance (P).

spectively; Fig. 3C is a higher magnification of a detail from Fig. 3B. It can be seen that in the control brain there is no positive avidin–biotin–peroxidase reaction (Fig. 3A), whereas in the brain perfused with IgG, both microvessel endothelial cells and the surrounding perivascular spaces show positive reactions (Figs. 3B and 3C), indicating passage of blood-borne IgG into the brain.

TABLE 1

Graphically Determined Unidirectional Transfer Constant K_{in} and Initial Volume of Distribution, V_i, for Homologous ^{125}I-IgG (2.5 μg/ml) Vascularly Perfused in the Guinea Pig Ipsilateral Forebrain

Brain region	n	K_{in} (ml min^{-1} g^{-1} × 10^3)	V_i (ml 100 g^{-1})	p (cm s^{-1} × 10^7)
		^{125}I-IgG		
Parietal cortex	10	0.58 ± 0.05*	0.29 ± 0.03	0.97 ± 0.08*
Caudate nucleus	10	0.53 ± 0.04*	0.29 ± 0.03	0.88 ± 0.07*
Hippocampus	12	0.55 ± 0.05*	0.28 ± 0.03	0.92 ± 0.08*
		[^{3}H]Dextran		
Parietal cortex	9	0.054 ± 0.010	0.31 ± 0.02	0.09 ± 0.02
Caudate nucleus	9	0.047 ± 0.010	0.30 ± 0.07	0.08 ± 0.02
Hippocampus	9	0.059 ± 0.010	0.29 ± 0.04	0.10 ± 0.02

Note. Cerebrovascular permeability constant, p, is calculated on the assumption of capillary surface area of 100 cm^2 (g brain)$^{-1}$ (2). Values are means ± SEM; * denotes $P < 0.001$ as compared with dextran K_{in} or p values, by analysis of variance.

TABLE 2

Blood–Brain Barrier Permeability to ^{125}I-IgG in Various Regions of Perfused Guinea Pig Brain in the Presence of Different Concentrations of Unlabeled IgG

IgG	Brain region	n	K_{in} (ml min^{-1} g^{-1} × 10^3)	V_i (ml 100 g^{-1})	% Change	P
0.06 mg/ml	Parietal cortex	9	0.56 ± 0.03	0.29 ± 0.03	−4	NS
	Caudate nucleus	9	0.49 ± 0.03	0.29 ± 0.02	−7	NS
	Hippocampus	9	0.51 ± 0.04	0.31 ± 0.04	−7	NS
4 mg/ml	Parietal cortex	6	0.13 ± 0.02	0.27 ± 0.03	−78	0.001
	Caudate nucleus	6	0.11 ± 0.02	0.29 ± 0.02	−79	0.001
	Hippocampus	6	0.13 ± 0.02	0.28 ± 0.02	−76	0.001

Note. Values are means ± SEM; the P value is the significance of percentage change of the unidirectional constant (K_{in}) for ^{125}I-IgG in the presence of different concentrations of unlabeled IgG compared to control values taken from Table 1.

DISCUSSION

The results of the present kinetic studies carried out over periods of up to 20 min (Fig. 1; Table 1) indicate a progressive time-dependent uptake of ^{125}I-IgG by the brain; the estimated unidirectional transfer constant of 0.53 to 0.58 ml min^{-1} g^{-1} × 10^3 was some 10 times greater than that measured for dextran (MW 70,000), a typical vascular marker. In the present experiments, the effective perfusion pressure was maintained at least 10 kPa below the threshold for a hypertensive opening of the BBB (2, 24). During guinea pig vascular brain perfusion, an opening of the BBB was possible to demonstrate only at perfusion pressures between 21 and 26 kPa (29). Thus, a significantly higher transfer rate of IgG in comparison to dextran studied under the same perfusion conditions cannot be attributed to pressure changes, or mechanical opening of the BBB to immune proteins.

Introduction of unlabeled IgG in the perfusate at low concentrations (0.06 mg/ml), that is, about 100 times less than the normal physiological plasma level of IgG in the guinea pig (34), had an insignificant effect. On the other hand, when higher concentrations of unlabeled IgG were included in the perfusate (4 mg/ml) there was a reduction in uptake of the label of 76 to 79% (Fig. 2; Table 2); or, if K_{in} for dextran is used as a background level, the self-inhibitory effect was 86 to 87%, indicating the presence of a saturable mechanism at the blood–brain barrier for the transport of IgG. Since the effective inhibitory concentration of unlabeled IgG was within the range of physiological plasma level of IgG in the guinea pig (34), the considerable inhibition obtained with 4 mg/ml IgG suggests a transport system that is already saturated at the physiological plasma level of IgG, i.e., therefore having an affinity constant, K_m, smaller than normal plasma concentration of IgG.

Immunohistochemical analysis of the brain tissue following perfusion with unlabeled IgG (Fig. 3) clearly showed a positive avidin–biotin–peroxidase reaction in both the endothelial cells of microvessels and the surrounding perivascular tissue, which was not possible to demonstrate in the control brains perfused with IgG-free medium. Immunoglobulin-containing cells have been previously shown in the brain tissue taken from patients with some inflammatory diseases of the nervous system (7), while elevated CSF indices for different classes of immunoglobulins and presence of immunoglobulin-producing cells in the CSF have been demonstrated in patients with an acute aseptic meningitis (14). On the basis of comparisons of CSF and plasma IgG and albumin levels, IgG synthesis within the CNS in patients with multiple sclerosis has been suggested (23). More recent work showed uptake of blood-borne IgG by retrograde axonal transport into the motor neurons projecting outside the BBB (8, 28), and differential intraneuronal accumulation of serum proteins has been considered to have possible implications for motor neuron diseases (8, 28). Data from the present study suggest that IgG may also be transferred across the BBB, supporting the hypothesis of a systemic IgG origin.

Saturable transport and transcytosis of the ^{125}I-labeled cationized form of IgG was recently demonstrated by isolated bovine microvessels, and passage of cationized ^{125}I-IgG through the BBB into the brain parenchyma was shown by thaw-mount autoradiography of frozen sections of rat brain obtained after carotid arterial infusions (25). The present study demonstrates, for the first time, transport of a native immune protein across the BBB and, moreover, suggests the presence of a saturable mechanism. The existence of such a saturable mechanism for transport of proteins at the BBB is in agreement with previous *in vitro* work with insulin (19), insulin-like growth factors (9), the cationized form of albumin (13), and transferrin (20), and supports the concept of carrier-mediated transport of these large molecules. This form of transport might well account for the presence of small concentrations of plasma proteins, including all the serum Ig classes, in the cerebrospinal fluid

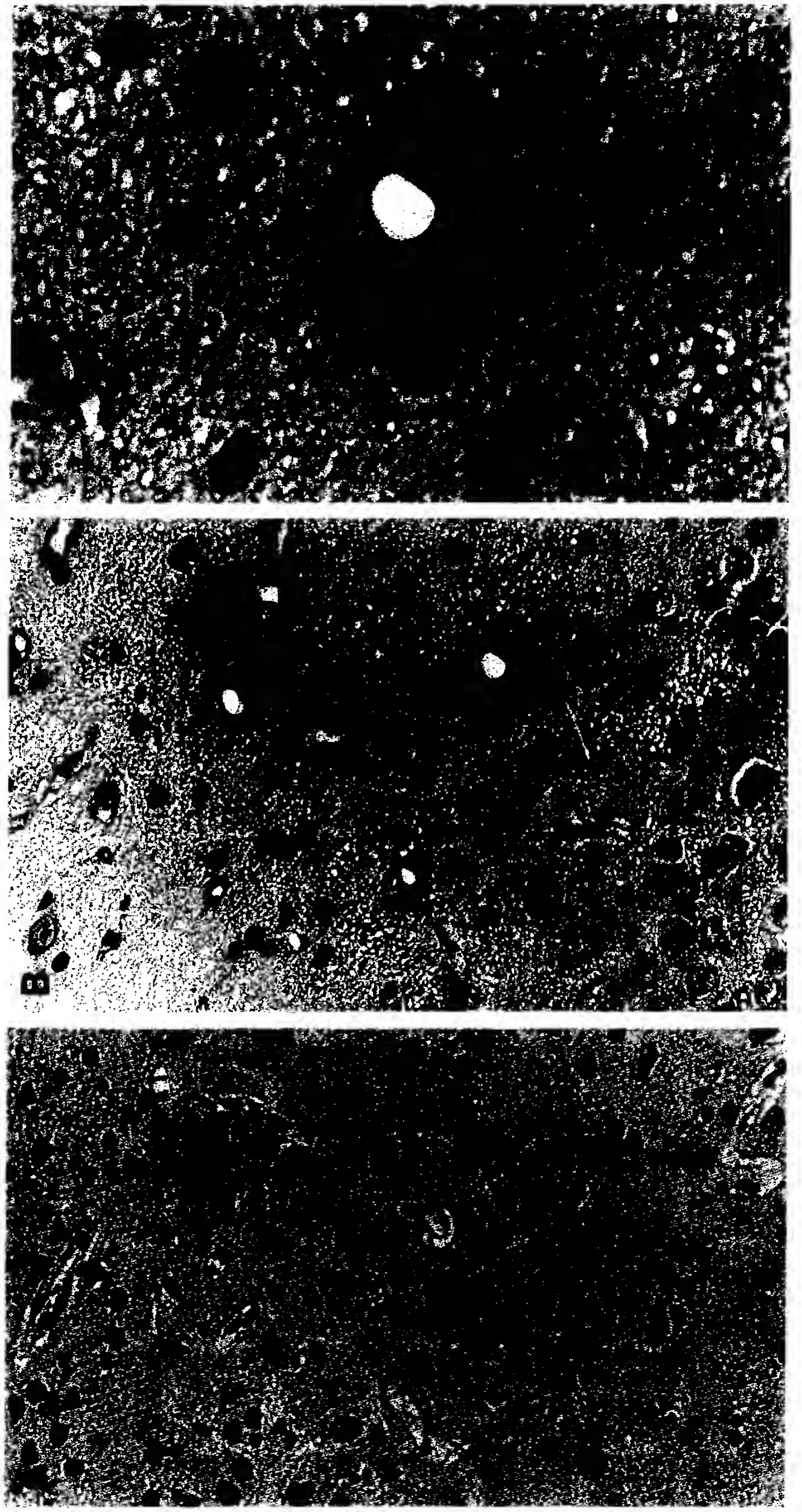

FIG. 3. (A) Negative avidin–biotin–peroxidase reaction in the parietal cortex perfused with IgG-free medium for 10 min, 242.5×. (B) Positive avidin–biotin–peroxidase reaction in the hippocampus perfused with unlabeled IgG (4 mg/ml) for 10 min, 388×. (C) Detail from B, 970×; reaction product is located at microvessel's walls and surrounding perivascular spaces. Arrows denote brain microvessels.

(5), although the possibility of protein brain synthesis cannot be excluded (6). This concept is also supported by recent findings of saturable mechanisms for peptides, such as delta-sleep-inducing peptide, both at the BBB and blood–CSF barrier, (31, 35) and leucin–enkephalin (30, 32, 36). It is thus clear that some naturally occurring peptides and proteins are, in fact, transported across the BBB, so that the failure to demonstrate significant passage of HRP from the brain capillaries (21, 4) should by no means discourage investigations of transport of peptides and proteins from blood to brain.

To conclude, the present study suggests that CNS may not be regarded as an immunologically privileged site, in so far as the possibility of blood-borne immunoglobins penetrating the brain is concerned. The fact that the transport system for IgG is saturated at normal physiological levels means that, normally, the brain is not supplied with immunoglobulins from the blood. Under certain immunodeficient conditions, however, with low serum IgG's, the transport system might well represent an additive protective mechanism. So far as therapeutic approaches to disease are concerned, this description of an apparent homeostatic mechanism controlling IgG levels in the brain might well be of value in supplementing the procedure of osmotic opening of the blood–brain barrier (10) and neural transplants (26, 22) as a means of delivering immune proteins to the CNS.

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TAB F

to
Second Declaration
of Kenneth L. Rock, M.D.,
dated May 19, 2008

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Immunocytochemical Localization of Immunoglobulins in the Rat Brain: Relationship to the Blood-Brain Barrier

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ABSTRACT

The central nervous system has been traditionally regarded as an immunologically privileged area. This feature has been in part attributed to the blood-brain barrier, which provides a restrictive interface to circulating immunoglobulins (IgG). Recent kinetic studies suggest that the barrier to immune proteins is not absolute, but rather may be regulated by a specific transfer mechanism. In this study, we confirm the presence of IgG in the central nervous system by immunocytochemistry and demonstrate a close anatomical relationship between the distribution of this protein and the blood-brain barrier.

IgG was immunolocalized in the normal rat brain by using monoclonal and polyclonal antibodies to IgG and its subclasses. On the basis of an initial evaluation, the most appropriate antibodies and dilutions were selected for subsequent analyses. In the first study, IgG and albumin were immunolocalized in adjacent sections. In the second study, horseradish peroxidase (HRP) was given intravenously prior to sacrifice, in order to examine artifacts related to perfusion fixation. The distribution of HRP and IgG was then examined in adjacent sections. In the third study, IgG was immunolocalized in sections of brain after mild traumatic head injury.

A monoclonal antibody to IgG2a and a polyclonal antibody to IgG were selected on the basis of specificity and consistent, mutual localization. Distinct, patchy, perivascular staining, infrequently associated with labeled neurons, was noted throughout the brain. Electron microsocopy confirmed the perivascular localization; IgG was localized along the basal lamina of microvasculature and within the adjacent parenchyma. Albumin and HRP did not exhibit a similar pattern of perivascular immunostaining. After head injury, prominent immunostaining for IgG was observed in the injured hemisphere.

In summary, these data indicate that the normal rat brain contains IgG, which dramatically increases after head injury. The distinct perivascular distribution in the normal brain suggests local microvascular permeability. This permeability is selective for IgG, since albumin does not share a similar perivascular localization. The neuronal staining which is closely associated with perivascular label may reflect one intracellular route for extravasated IgG. © 1994 Wiley-Liss, Inc.*

Key words: immunocytochemistry, immunoglobulin G, neuronal accumulation of protein

The blood-brain barrier consists of specialized endothelial cells that restrict and selectively regulate transport between the plasma and neural compartments. The restrictive features of the blood-brain barrier relate to the presence of tight junctions between endothelial cells and the limited expression of nonreceptor-mediated transport across the cell (Reese and Karnovsky, 1967; Brightman et al., 1970). These combined features provide the basis for the blood-brain barrier to lipid-insoluble macromolecules.

Recent experiments in our laboratory (Tanno et al., 1992a,b), as well as others (Triguero et al., 1989; Zlokovic et al., 1990), have raised a question about the relative permeability of the blood-brain barrier to circulating IgG. In the course of examining the blood-brain barrier to IgG after experimental head injury, we noted that brains from the surgical control group exhibited a distinct perivascular pattern of immunostaining. This localization did not have

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any consistent anatomical relationship to the site of craniotomy or to areas of the brain considered to be outside the blood-brain barrier. One explanation for this perivascular distribution is that cerebral microvasculature may exhibit some degree of permeability to IgG. Studies by Triguero et al. (1989) and Zlokovic et al. (1990) support such an hypothesis. These investigators demonstrated transport of isotopically labeled IgG across brain vasculature. Taken together, these findings are of particular interest since they challenge the traditional view of the blood-brain barrier and raise an intriguing question about the extent to which the brain is immunologically privileged.

In the present study, we examined the blood-brain barrier to IgG at the light and ultrastructural levels. In addition, we focused on potential artifacts that could contribute to a "false positive" pattern of immunostaining. The present studies confirm the distinct perivascular immunolocalization of the protein and detail its ultrastructural localization.

MATERIALS AND METHODS

Surgical procedures

Adult male Sprague-Dawley rats (350–400 g) were used for these studies. All animals were anesthetized with 4.0% chloral hydrate (8–10 ml/kg, intraperitoneally [i.p.]).

Uninjured group. Rats were perfused with 500 ml of paraformaldehyde (PFA) (4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 [PB]) or periodak-lysine-paraformaldehyde (PLP) (2.0% paraformaldehyde and 0.01 M $NaIO_4$ in buffered lysine, pH 7.4). Brains were postfixed for 4 hours. Each brain was divided into 5 regions corresponding to the following levels: caudate putamen, thalamus, lateral geniculate nucleus, superior colliculus, and midcerebellum. Serial sections, 100 μm thick, were cut on a vibratome. Sections were floated in wells containing 0.05 M phosphate-buffered saline, pH 7.4 (PBS). Adjacent sections were collected for immunocytochemical staining and controls.

Injured group. Rats were prepared for lateral fluid percussive brain injury as described previously (Ishige et al., 1987). Briefly, rats were anesthetized as above and after incision of the temporal muscle, a circular craniotomy, 4 mm in diameter, was made with a dental drill just above the zygoma. A polyethylene tube (PE 350) filled with isotonic saline was placed against the intact dura, fixed securely to the skull with dental acrylic cement, and connected to a fluid percussive device. A catheter was inserted into the femoral artery to monitor blood pressure. Each animal was then subjected to an impact pressure of 4.8–5.2 atm for 20 msec. The animals breathed spontaneously throughout the whole procedure. Normal body temperature was maintained with a water-circulating heating pad.

At 24 hours postinjury, animals were anesthetized and perfused with either PFA or PLP. The brains were removed and prepared according to the procedure described for the uninjured group.

Immunolocalization of IgG and albumin

Antibody evaluation. IgG was immunolocalized in the normal rat brain by using monoclonal and polyclonal antibodies to IgG. Fourteen animals were used for this study; each antibody was used for immunostaining in 1–4 animals. Specific and reliable antibodies were identified in an initial screening and used for subsequent studies. Detailed parameters for the immunocytochemical localization of IgG are presented in Table 1.

TABLE 1. Parameters for the Immunocytochemical Localization of IgG in the Brain Obtained by the Use of Polyclonal (Pab) and Monoclonal (Mab) Antibodies

IgG type	Antibody	Vendor	Concentration range, mg/ml	Optimal concentration, mg/ml
IgG	Pab[1]	Vector[3,4]	0.0008–0.03	0.0015
IgG	Pab[2]	Vector	0.00025–0.01	0.0005
IgG-2a	Pab	Binding Site[5]	0.0005–0.02	0.001
IgG-1	Mab	Caltag[6]	0.0002–0.008	0.008
IgG-1	Mab	Zymed[6]	0.0005–0.02	*
IgG-2a	Mab	Caltag	0.0002–0.016	0.016
IgG-2a	Mab	Zymed[3]	0.0005–0.02	0.01
IgG-2b	Mab	Caltag	0.0002–0.008	*
IgG-2c	Mab	Caltag	0.0002–0.008	*
IgG kappa	Mab	Caltag	0.0002–0.008	*
IgG kappa	Mab	Zymed	0.0005–0.02	*
IgG lambda	Mab	Zymed	0.0005–0.02	*

[1]Biotinylated.
[2]Biotinylated, mouse-adsorbed.
[3]Selected for final analysis.
[4]Burlingame, CA.
[5]Birmingham, England.
[6]S. San Francisco, CA.
*No obvious staining.

Albumin was immunolocalized in the normal rat brain (n = 3) by means of two polyclonal antibodies. One antibody that exhibited low background staining and could be effectively blocked with purified antigen was selected for further studies. Immunolocalization of albumin and IgG was then examined in adjacent sections of normal brain (n = 1).

Peroxidase quenching. Sections were incubated in 1.0% H_2O_2 in PBS for 10 minutes, to quench any endogenous peroxidatic activity, then rinsed in PBS. All incubations were carried out at room temperature.

IgG, polyclonal. Sections were incubated in each one of the following solutions for the time indicated: 2.0% rabbit serum/0.2% Triton X-100/0.1% bovine serum albumin (RS/TX/BSA), 5 minutes; 10.0% rabbit serum/0.2% Triton X-100/0.1% bovine serum albumin (10%RS/TX/BSA), 20 minutes; biotinylated rabbit anti-rat IgG or biotinylated rabbit anti-rat IgG, mouse adsorbed, in RS/TX/BSA, 2 hours; PBS, 3 × 10 minutes.

IgG2a, polyclonal. Sections were incubated in each one of the following solutions for the time indicated: RS/TX/BSA, 5 minutes; 10% RS/TX/BSA, 20 minutes; sheep anti-rat IgG2a, in RS/TX/BSA, 2 hours; PBS, 3 × 10 minutes; biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA; 0.0075 mg/ml) in RS/TX/BSA, 1 hour; PBS, 3 × 5 minutes.

IgG, monoclonal. Sections were incubated in each one of the following solutions for the time indicated: 2.0% horse serum/0.2% Triton X-100/0.1% bovine serum albumin (HS/TX/BSA), 5 minutes; 10.0% horse serum/0.2% Triton X-100/0.1% bovine serum albumin (10%HS/TX/BSA), 20 minutes; mouse anti-rat antibody (directed against IgG1, IgG2a, IgG 2b, IgG2c, IgG kappa chain, or IgG lambda chain) in HS/TX/BSA, 2 hours; PBS, 3 × 10 minutes; biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories, Burlingame, CA; 0.0025 mg/ml) in HS/TX/BSA, 1 hour; PBS, 3 × 5 minutes.

Albumin, polyclonal. Sections were incubated in each one of the following solutions for the time indicated: 0.5% casein in 0.05% PBS (CA/PBS), 20 minutes; sheep anti-rat albumin (Cappel Research Products, Durham, NC; 0.0008–0.16 mg/ml) or goat anti-rat albumin (Nordic Immunology, Capistrano Beach, CA; 0.0017–.02 mg/ml) in CA/PBS,

hours; PBS, 3 × 10 minutes; biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA; 0.0075 mg/ml) in CA/PBS, 1 hour; PBS, 3 × 5 minutes.

Method of detection. All sections were subsequently incubated in Avidin-Biotin-HRP complex (ABC; Vector Laboratories, 1:100, in PBS) for 30 minutes and the final reaction product was visualized with 0.05% 3,3-diaminobenzidine tetrachloride (DAB) as the chromogen in the presence of 0.02% H_2O_2. The sections were rinsed in PBS, mounted on gelatinized slides, dehydrated in graded alcohols, cleared in Hemo-De (Fisher Scientific, Santa Clara, CA), and coverslipped. Immunostaining of IgG was then evaluated by light microscopy.

Immunocytochemical controls. As a control for nonspecific staining, sections were stained as outlined above, with the omission of the primary antibody.

Preadsorption controls were completed for the polyclonal antibodies IgG, IgG, mouse adsorbed, IgG2a, and albumin (Nordic and Cappel) and the monoclonal antibody against IgG2a. Sections were stained as outlined above, but the primary antibody was incubated for 30 minutes with purified protein (0.05 mg/ml) prior to addition to the sections.

As a control for species specificity, as well as to assess nonspecific staining, sections were stained as described above, with mouse anti-human IgG (Dako, Glostrup, Denmark; 0.0005–0.02 mg/ml) as the primary antibody.

Ultrastructural immunolocalization of IgG

IgG was immunolocalized in the normal rat brain (n = 2) with a polyclonal antibody. The rats were anesthetized and perfused with 0.025% glutaraldehyde, 4.0% paraformaldehyde in PB. The brains were removed and prepared according to the procedure described above. Sections were immunostained with polyclonal biotinylated rabbit anti-rat IgG, according to the procedure described above. Serial sections, 100 μm thick, were examined with a light microscope and immunostained regions of cortex, hippocampus, and cerebellum were excised for electron microscopic analysis. Excised pieces were rinsed in PBS, postfixed for 2 hours in 1.0% osmium tetroxide in PB, and rinsed again in PBS. They were then dehydrated in graded alcohols and dimethoxypropane and embedded in Araldite 502. Sections, 60 nm thick, were cut on an ultramicrotome and mounted on 200 mesh uncoated copper grids. The sections were stained with lead citrate and examined and micrographed with a JEOL 100S electron microscope.

Localization of horseradish peroxidase (HRP) and IgG

Adjacent sections were examined for vascular permeability to IgG and HRP. Animals (n = 2) were anesthetized and given diphenhydramine hydrochloride (10 mg in 0.2 ml, i.p.) to block any histamine release. HRP (Type II; 0.15 mg/kg, i.v.; Sigma, St. Louis, MO) was administered 10 minutes prior to euthanasia. The rats were perfused with PFA and the tissue was prepared according to the procedure described above. Sections were divided into four groups: (1) to visualize HRP, sections were incubated in 0.05% DAB and 0.02% H_2O_2; (2) to determine whether endogenous and/or injected peroxidase could be effectively quenched, sections were incubated in 1.0% H_2O_2 for 10 minutes and then incubated in DAB and H_2O_2; (3) to visualize IgG, sections were quenched as described above and then stained with biotinylated Pab IgG (0.03 mg/ml) as described above; and (4) as a control for nonspecific secondary antibody staining, the same procedure was performed as in (3) with the omission of the primary antibody.

Immunolocalization of IgG in the injured brain

IgG was immunolocalized in the rat brain after mild head injury (n = 4). On the basis of light microscopic screening of antibodies in the uninjured brain, polyclonal anti-rat IgG and monoclonal anti-rat IgG2a were selected for this study (refer to Table 1). The rats were anesthetized, perfused, and the tissue prepared according to the procedure described above. IgG and IgG2a were immunolocalized in adjacent sections of brain according to the procedure outlined above.

Immunocytochemical controls. Nonspecific secondary and preadsorption controls were as described for the IgG studies.

RESULTS

A variety of monoclonal and polyclonal antibodies directed against IgG and its subtypes were screened and a dilution series was run for each antibody. Immunostaining for IgG was then examined using the most specific antibodies at the optimal dilutions.

Immunocytochemical controls

No staining was associated with any of the controls except for albumin, as noted below.

Antibody evaluation

The optimal concentration of each antibody was based upon consistent specific staining in nonblood-brain barrier regions (choroid plexus, subfornical organ, median eminence, and area postrema) and brainstem nuclei (trigeminal motor nucleus, facial nucleus). Most of the monoclonal antibodies yielded negative staining and when staining was observed, it was inconsistent. No differences in immunostaining were observed if the polyclonal antibody was adsorbed against mouse serum. The most consistent staining was found with the monoclonal and polyclonal antibodies against IgG2a (Mab IgG2a and Pab IgG2a) and the polyclonal antibody against IgG (Pab IgG, Fig. 1). Given these consistent findings, Pab IgG and Mab IgG2a were selected for further analysis.

Immunolocalization of IgG and albumin

Albumin was immunolocalized in the normal rat brain using two polyclonal antibodies to the protein. Considerable nonspecific staining was noted with the sheep anti-rat albumin at high concentrations and preadsorption with the protein did not block this staining. The goat anti-rat albumin was more specific; minimal background staining was noted and preadsorption controls were negative. Based upon these findings, goat anti-rat albumin was selected for further studies to examine the relationship between albumin and IgG in the brain.

Adjacent sections of one animal were immunostained for IgG and albumin (Fig. 2). Sections were examined at the level of the caudate putamen, thalamus, superior colliculus, and midcerebellum. Both serum proteins were consistently noted in those areas of the brain considered outside the blood-brain barrier, including choroid plexus, subfornical organ, median eminence, and area postrema. Typically, an

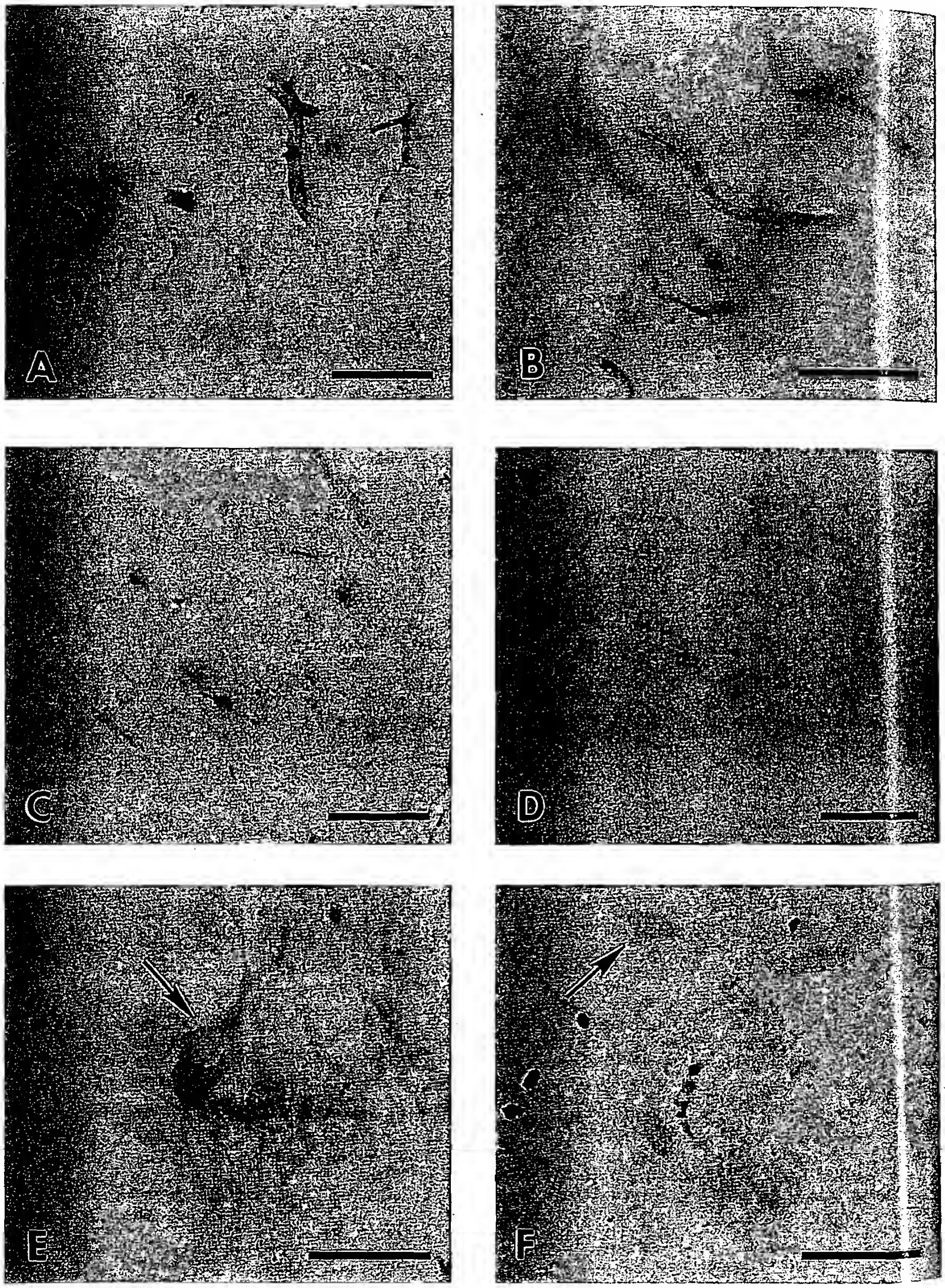

Figure 1

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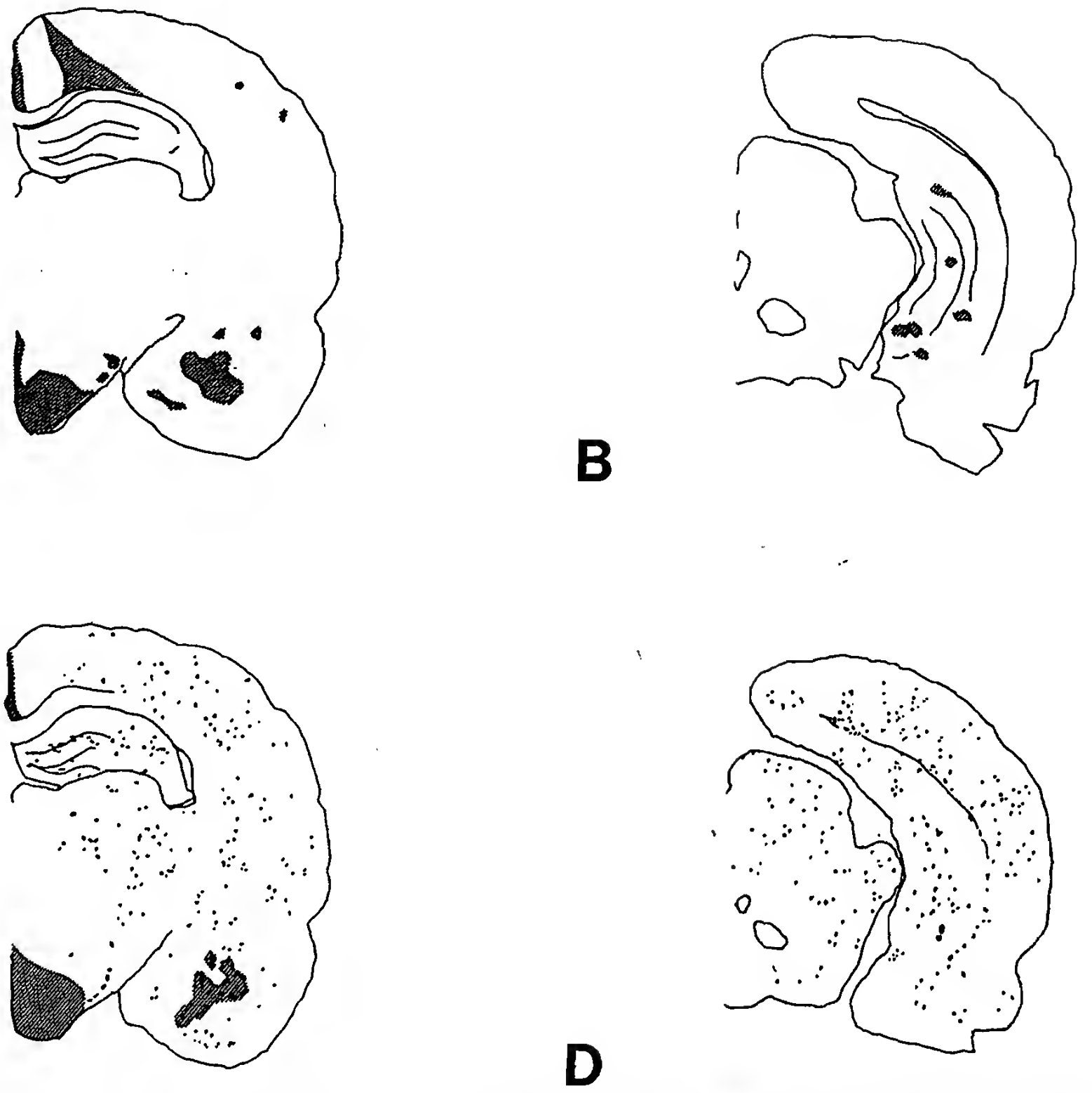


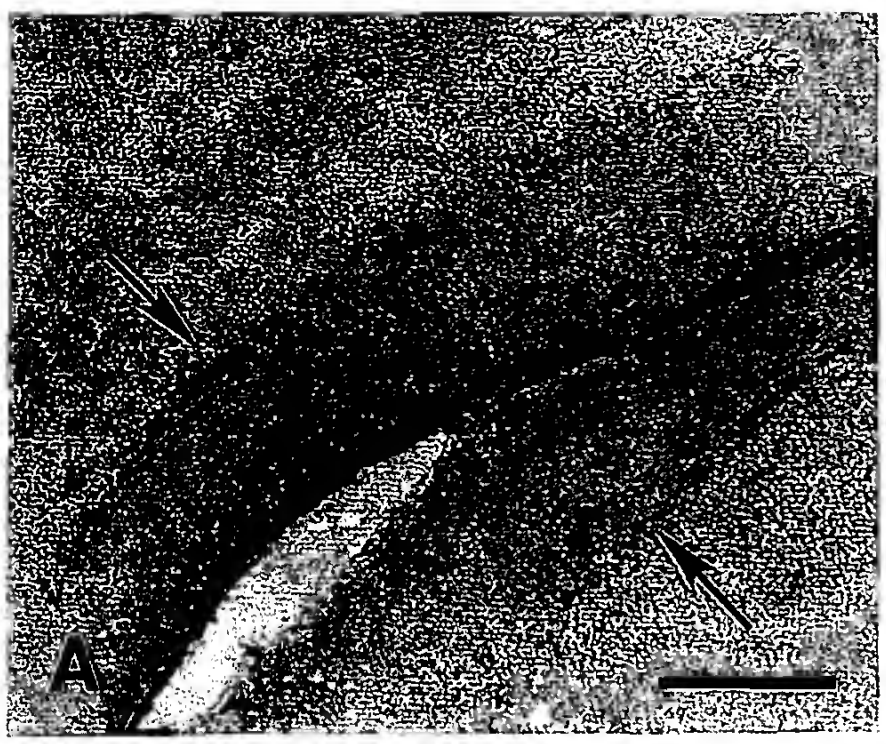
Fig. 2. A comparison of the distribution of IgG and albumin in the normal brain. Tracings of adjacent sections of brain immunostained with Pab IgG (0.03 mg/ml) or Pab albumin (0.00167 mg/ml). **A, B:** Albumin is localized in a diffuse pattern that is relatively restricted in its distribution (hatched areas). **C, D:** In contrast, IgG is typically localized to distinct perivascular cuffs throughout the brain (stipples) and exhibits a more limited distribution that is diffuse in appearance (hatched areas).

intense diffuse immunostain was noted and neurons were frequently labeled. Both proteins were also immunolocalized in motor neurons in brainstem nuclei including the trigeminal motor nucleus, facial nucleus, and hypoglossal nucleus.

Distinct differences in the pattern of immunostaining were noted for IgG and albumin (Fig. 2). In general, IgG was widespread with distinct perivascular cuffs. In contrast, albumin was restricted to regions that exhibited a light, diffuse pattern of immunostaining. Both proteins were immunolocalized in neurons (Figs. 3,4). With few exceptions, these labeled neurons were associated with regions that also exhibited either a diffuse or perivascular pattern of immunostaining.

IgG was immunolocalized in scattered perivascular cuffs throughout the cingulate, frontoparietal, retrosplenial, occipital, entorhinal, and piriform cortices (Figs. 1,2). Similar staining was also noted in the caudate putamen, amygdala, thalamus, hippocampus, superior colliculus, and medial geniculate nucleus. Immunostained neurons were closely

Fig. 1. Immunolocalization of IgG. Specificity of perivascular and neuronal labeling. **A:** Pab IgG (0.03 mg/ml) is localized in distinct perivascular cuffs. **B:** At higher magnification, immunostaining is associated with the vascular walls and the surrounding tissue. **C:** Further dilution of Pab IgG to 0.0015 mg/ml does not eliminate perivascular immunoreactivity in the frontoparietal cortex. **D:** Similar perivascular immunolabel is also apparent with Mab IgG2a (0.001 mg/ml). Neurons whose processes project into the periphery (E, trigeminal nucleus, arrow) or terminate in brain regions considered to be outside the blood-brain barrier (F, nucleus of the solitary tract, arrow) are typically immunolabeled with Pab IgG (E, 0.0015 mg/ml) and Mab IgG2a (F, 0.001 mg/ml). Bars = 100 μm in A, C, and D, 25 μm in B, E, and F.



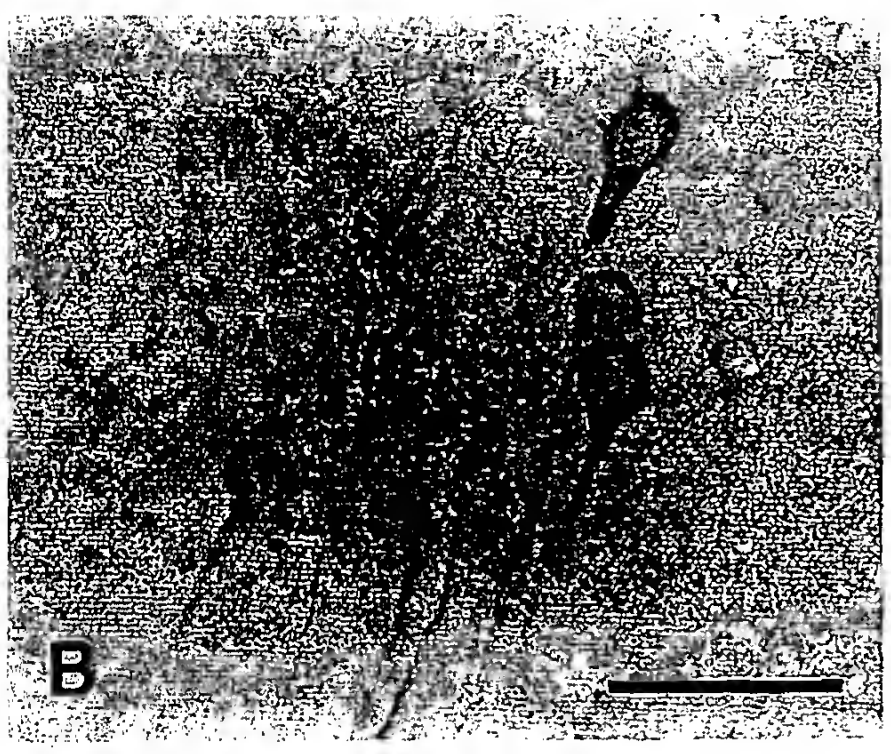
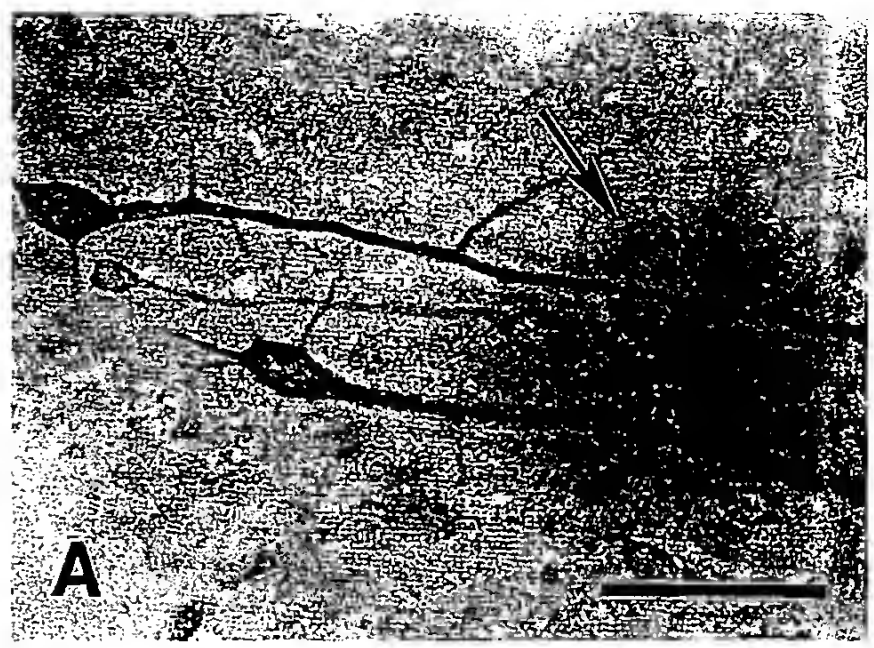


Fig. 3. Neurons immunolabeled with Mab IgG2a (0.001 mg/ml). **A:** Immunolabeled Purkinje cells are apparent (arrows). In addition, discrete bands of label, presumably reflecting immunolabeled cellular processes, extend from the cerebellar surface toward the Purkinje cell layer. **B:** Perivascular immunostaining in the frontoparietal cortex is intimately associated with immunolabeled neurons. Bars = 200 μm in A, 50 μm in B.



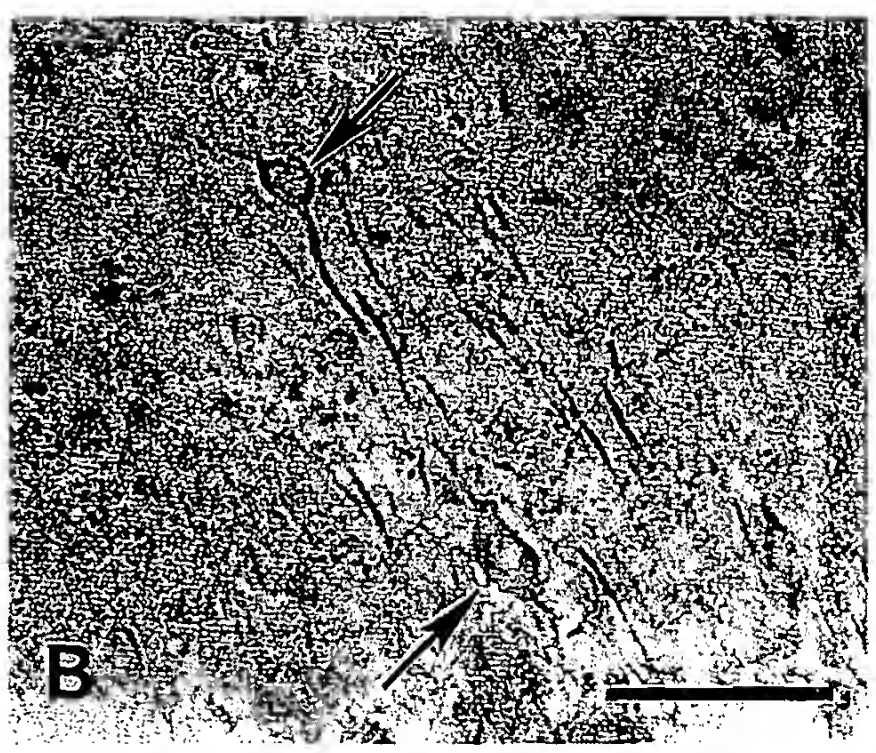


Fig. 4. The typical appearance of frontoparietal neurons immunolabeled for IgG or albumin. **A:** Pab IgG (0.03 mg/ml) is immunolocalized in neurons and their processes. These processes course through an intensely labeled zone (arrow). **B:** Neurons, immunolabeled with Pab albumin (0.00167 mg/ml), appear only lightly stained (arrows) and have no obvious affiliation with blood vessels. Bars = 50 μm.

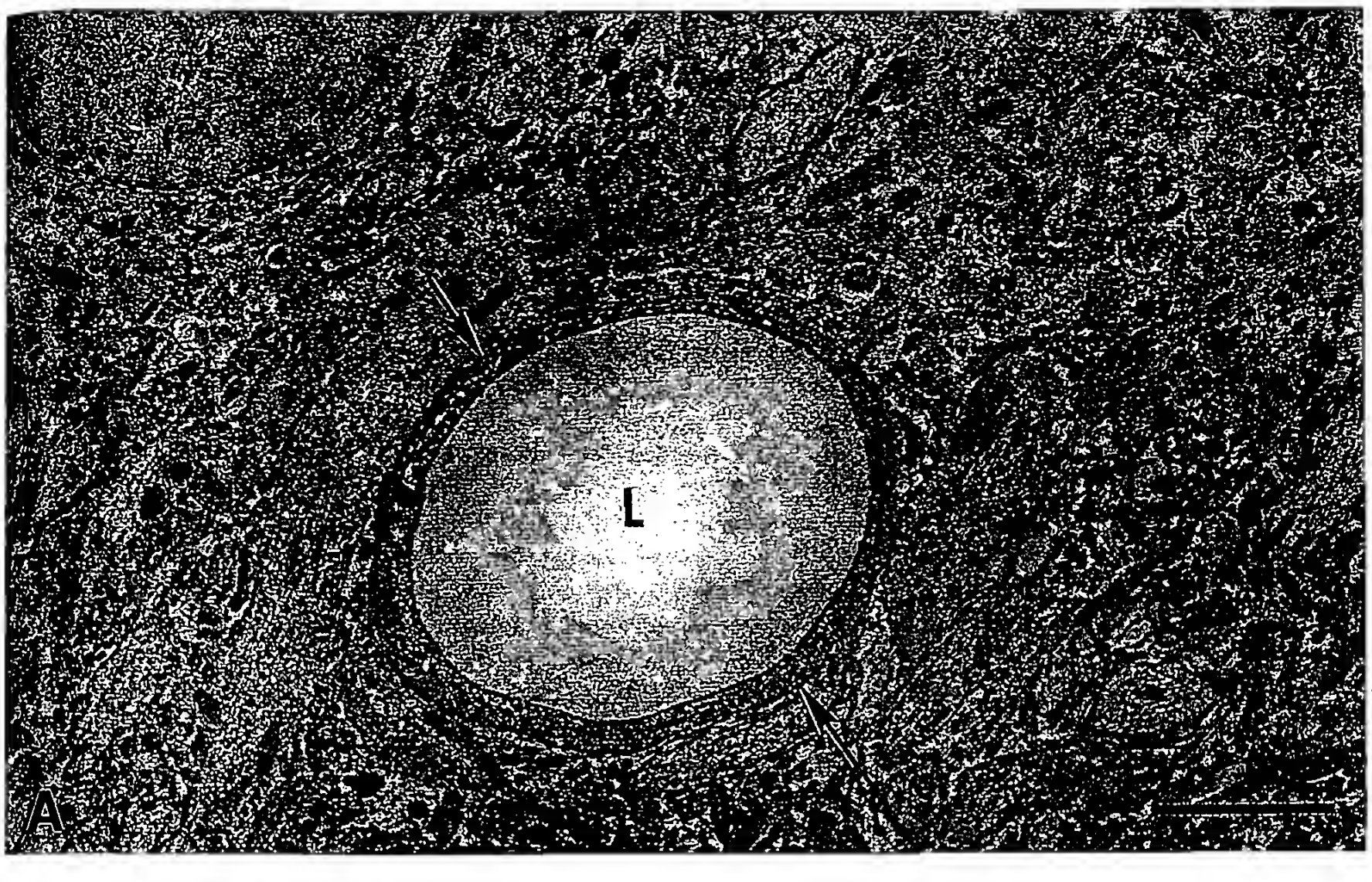
associated with this perivascular immunostaining. This relationship between neurons and vessels was most consistently observed in the cortex and to a lesser extent in the hippocampus and thalamus (Figs. 3,4).

Immunostaining for albumin was rarely seen in perivascular cuffs, but instead in diffuse patches (Figs. 2,4). This diffuse immunostaining was most apparent in the frontoparietal, cingulate, and piriform cortices, and caudate putamen. Very light immunostaining was noted in the hippocampus. Lightly immunostained neurons were most evident in the frontoparietal and cingulate cortices (Fig. 4). There was no obvious relationship between these immunostained neurons and the vasculature.

In the cerebellum and brainstem, IgG and albumin shared a similar immunolocalization. Immunostaining was noted in the subarachnoid space and in Purkinje cells in the adjacent cerebellar cortex (Fig. 3).

Ultrastructural immunolocalization of IgG

Immunoelectron microsocopy was used to more precisely define the perivascular distribution of IgG. In order to preserve antigenicity, the concentration of glutaraldehyde in the fixative was reduced; as a result, preservation of neuronal and glial ultrastructure was not optimal. Nevertheless, the vascular architecture was well perserved. Reaction product was distinctly localized to the basal lamina of capillaries, arterioles, and venules (Figs. 5–7). Various components of the Virchow-Robin spaces were also labeled; these included the basal lamina of the glia limitans and leptomeningeal cells (Fig. 5). The parenchyma adjacent to immunolabeled vessels also contained a patchy distribution of reaction product (Fig. 7a, closed arrowhead). However, the indistinct cytoarchitectural detail precluded more defined localization.



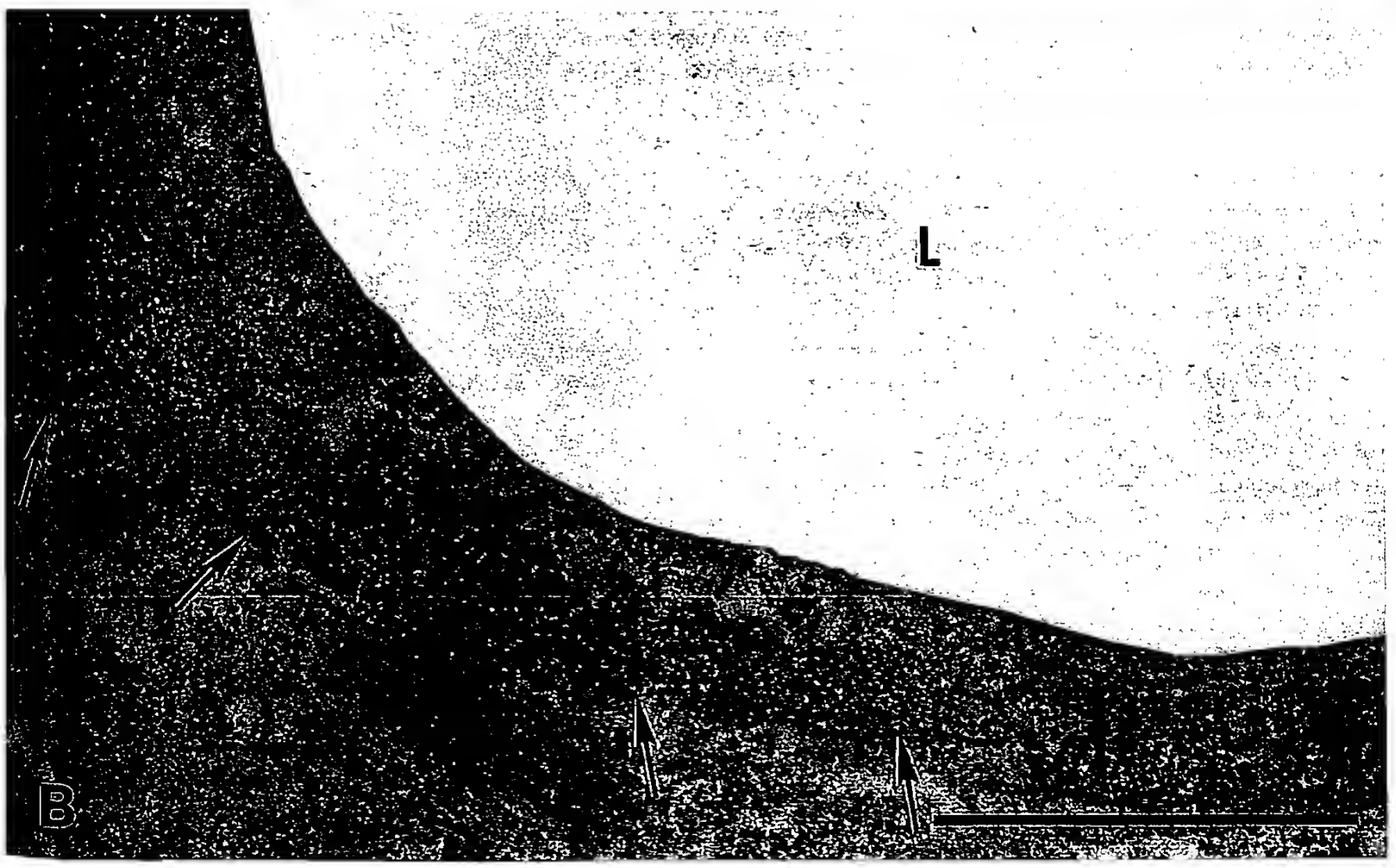


Fig. 5. Immunolocalization of Pab IgG (0.03 mg/ml) in a cortical capillary. **A:** Reaction product is localized in a perivascular distribution (arrows). **B:** Higher magnification. IgG is localized adjacent to the endothelial cell in a structure with an irregular contour (arrows). The irregular contour and thickness of this labeled structure and proximity to the endothelial cell suggest an astrocytic process or possibly a pericyte. L, lumen. Bars = 2 μm in A, 1 μm in B.

Localization of HRP and IgG

Permeability to the exogenous tracer HRP was evaluated in uninjured animals. Alternate sections were prepared for HRP histochemistry or IgG immunocytochemistry. HRP was restricted to nonblood-brain barrier regions (Fig. 8). In contrast, perivascular immunostaining for IgG was evident as previously described. Detailed differential localization was noted using darkfield optics (Fig. 9). Circumferential rings of immunostaining, presumably labeling the smooth muscle coat, were noted in arterioles. A similar pattern of staining was not observed for HRP.

Immunolocalization of IgG in the injured brain

Immunostaining with polyclonal antibody (Pab) IgG and monoclonal antibody (Mab) IgG2a was performed on adjacent sections of injured tissue. The pattern of staining was similar for both antibodies, although, as would be expected, the stained regions were more restricted and the stain was less intense with the monoclonal antibody (Fig. 10).

The regions that were immunostained in the normal animals also stained in injured animals, with diffuse staining in nonblood-brain barrier regions and around scattered vessels. In addition, intense diffuse and cellular immunostaining was found in the injured hemisphere.

At the impact site, hemorrhage was restricted to the external capsule. Immunostained regions were seen surrounding the hemorrhage, in the superficial and deep layers of the frontoparietal cortex, in the retrosplenial and cingulate cortices, hippocampus (hippocampal fissure, CA1–3), caudate putamen, and superior colliculus.

Immunostained neurons and glia were prominent in the regions surrounding the impact site, in the oriens and pyramidal cell layers of the dorsal region of the hippocampus, and in the superior colliculus. These neurons were frequently localized in regions that also exhibited intense diffuse immunostaining. Typically, the cell bodies as well as processes were immunolabeled (Fig. 11).

DISCUSSION

The blood-brain barrier to plasma proteins has been attributed to the physical restriction imposed by tight junctions, adjoining endothelial cells, and the lack of an effective transcellular route (Reese and Karnovsky, 1967; Brightman et al., 1970). The findings presented in this study support the concept that the blood-brain barrier is not absolute, but rather may exhibit discrete permeability changes. How this might occur remains speculative.

An important methodologic concern in this study was to establish appropriate guidelines for specific immunolocalization of IgG in the brain. There are several important considerations for accurate and specific immunolocalization of protein. Specificity of the primary antibody should be confirmed and immunostaining should be restricted to the appropriate antigenic sites. Preadsorption controls provide one line of evidence for specificity of antibodies. In our study, immunostaining was effectively blocked when sections of tissue were incubated with primary antibody that had been first exposed to purified antigen. In order to minimize nonspecific staining but maintain an accurate signal, criteria for an appropriate dilution of the primary antibody should be established. In preliminary studies, we varied the dilution of the primary antibody and examined the intensity of immunoreactivity in known permeable regions of the brain including the choroid plexus, meninges, and area postrema. Brainstem nuclei were also included in this survey because there is an extensive body of literature that supports the view that these neurons accumulate plasma proteins via retrograde transport (Kristensson et al., 1971; Broadwell and Brightman, 1976; Sparrow, 1981; Balin et al., 1986; Fabian and Petroff, 1987; Yamamoto et al., 1987; Moos et al., 1991). At all concentrations, we found that the choroid plexus and meninges stained more intensely than brainstem nuclei. Because of this differential response, very dilute concentrations of antibody produced no staining in brainstem nuclei. Based upon these results, we selected the lowest concentration of primary antibody that would be sufficiently sensitive to detect IgG in brainstem nuclei.

It is possible that impurities may have been present during the production of the Pab IgG, resulting in the generation of unwanted antibodies. This concern was indirectly assessed by comparing the immunolocalization of IgG and its major subclass, IgG2a, using both polyclonal and monoclonal antibodies. As might be expected, the intensity of immunostaining varied such that Pab IgG > Pab IgG2a > Mab IgG2a. This diminution most likely reflected the concentration of available binding sites. Despite this variation in intensity of immunostaining, a consistent pattern of immunolocalization was noted in adjacent sections of brain, a finding that further confirms the specificity of our immunologic probes.

As a final test of specificity, we examined the anatomical pattern of breakdown of the blood-brain barrier after experimental brain trauma, using Pab IgG and Mab IgG2a. We have previously demonstrated that this injury produces marked barrier breakdown to an exogenous protein tracer and that sections of brain exhibit enhanced immunoreactivity to Pab IgG (Tanno et al., 1992a,b). In the present report, we found that there was enhanced immunostaining, as detected with both the monoclonal and polyclonal antibodies, in regions of the brain that typically exhibit barrier breakdown after brain trauma. Furthermore, there was a close anatomical relationship between immunostaining for Pab IgG and Mab IgG2a in the injured hemisphere. These findings add further credibility to the specificity of our immunocytochemistry.

Studies that have examined the permeability of the blood-brain barrier have yielded conflicting results. Seitz et al. (1985) and Azzi et al. (1990) reported that the blood-brain barrier is impermeable to IgG. In contrast, in the present study we noted a consistent perivascular immunoreactivity to Pab IgG. It has been suggested that such a localization may reflect an artifact of perfusion fixation or inadequate tissue fixation (Sparrow, 1980; Balin et al., 1986; Fabian and Petroff, 1987; Broadwell and Sofroniew, 1993). Artifactual permeability may result from either the rupture of vessels during perfusion fixation or a delay in adequate fixation after euthanasia. Sparrow (1980) hypothesized that perivascular immunolocalization of serum proteins was due to their artifactual diffusion during the preparation of frozen sections. Given these concerns, we focused on the issue of fixation artifact as a possible cause of perivascular immunolocalization of IgG.

To address the possibility that perfusion fixation disrupts blood vessels, we compared the permeability of the blood-

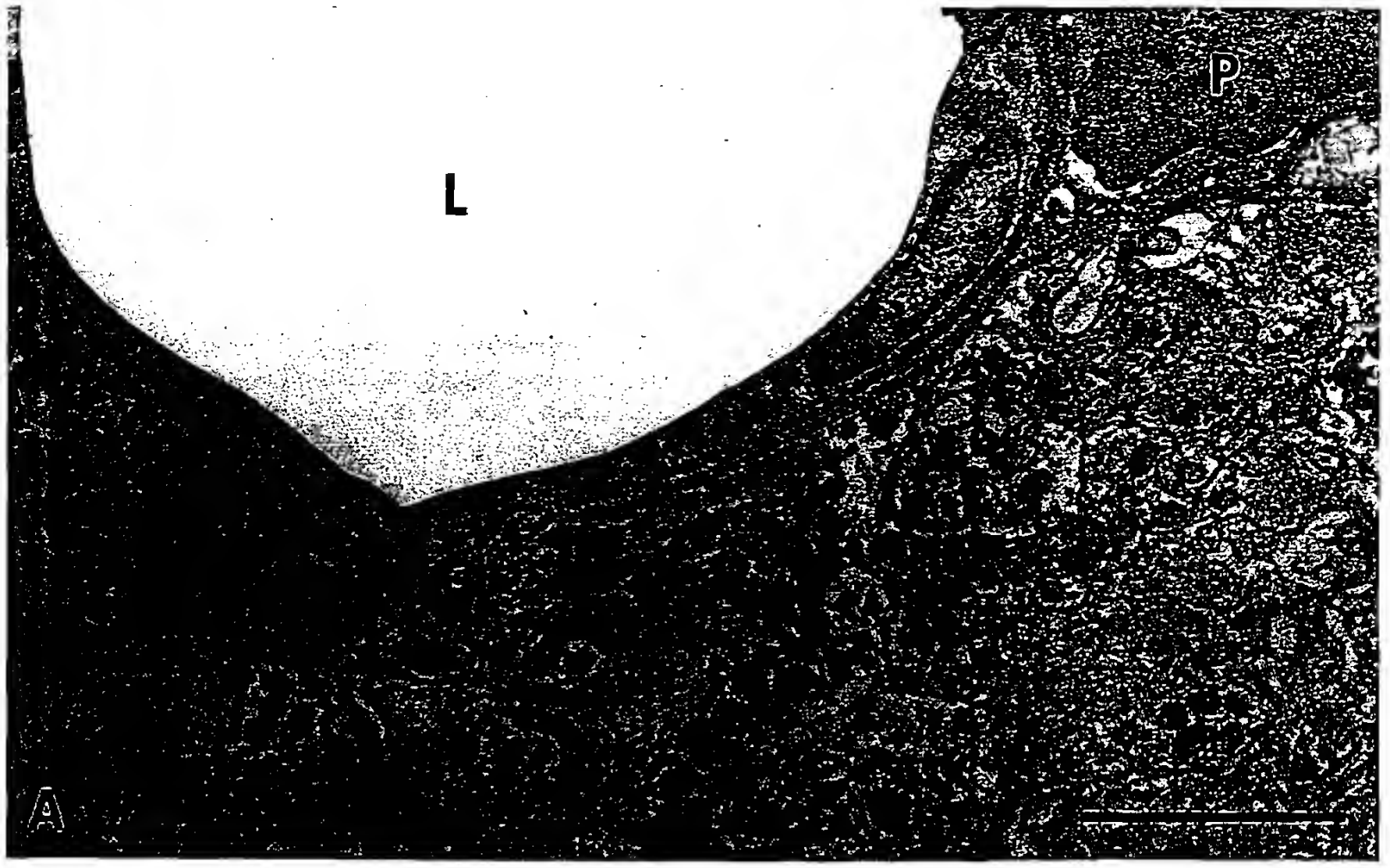


Fig. 6. **A:** Immunolocalization of Pab IgG (0.03 mg/ml) in a cortical arteriole. **B:** Higher magnification. Reaction product clearly delineates the basal lamina (arrows) that splits to enclose a pericyte (P). No reaction product is apparent in the interstitium. L, lumen. Bars = 2 μm in A, 1 μm in B.

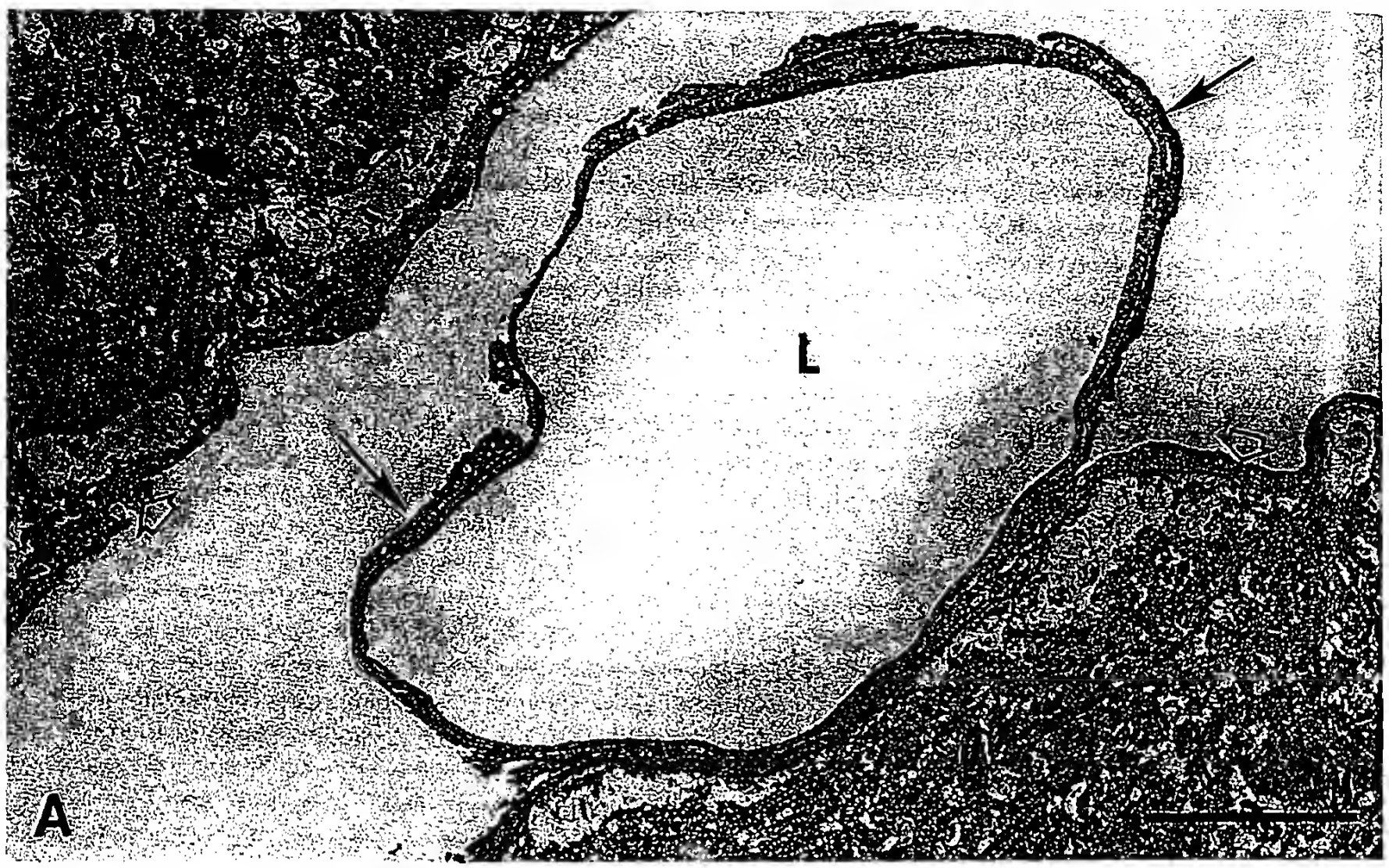


Fig. 7. Immunolocalization of Pab IgG (0.03 mg/ml) along a thin-walled vessel, presumably a venule, in the cerebellar subarachnoid space. A: Reaction product is apparent along the abluminal aspect of the vessel (arrows), is associated with the glia limitans (open arrowheads) and is present in the parenchyma (closed arrowhead). B: Higher magnification. Immunolabel intervenes between the vascular basal lamina (open arrowheads) and the basal lamina (arrows) associated with the adjacent parenchyma (PA). L, lumen. Bars = 5 μm in A, 1 μm in B.

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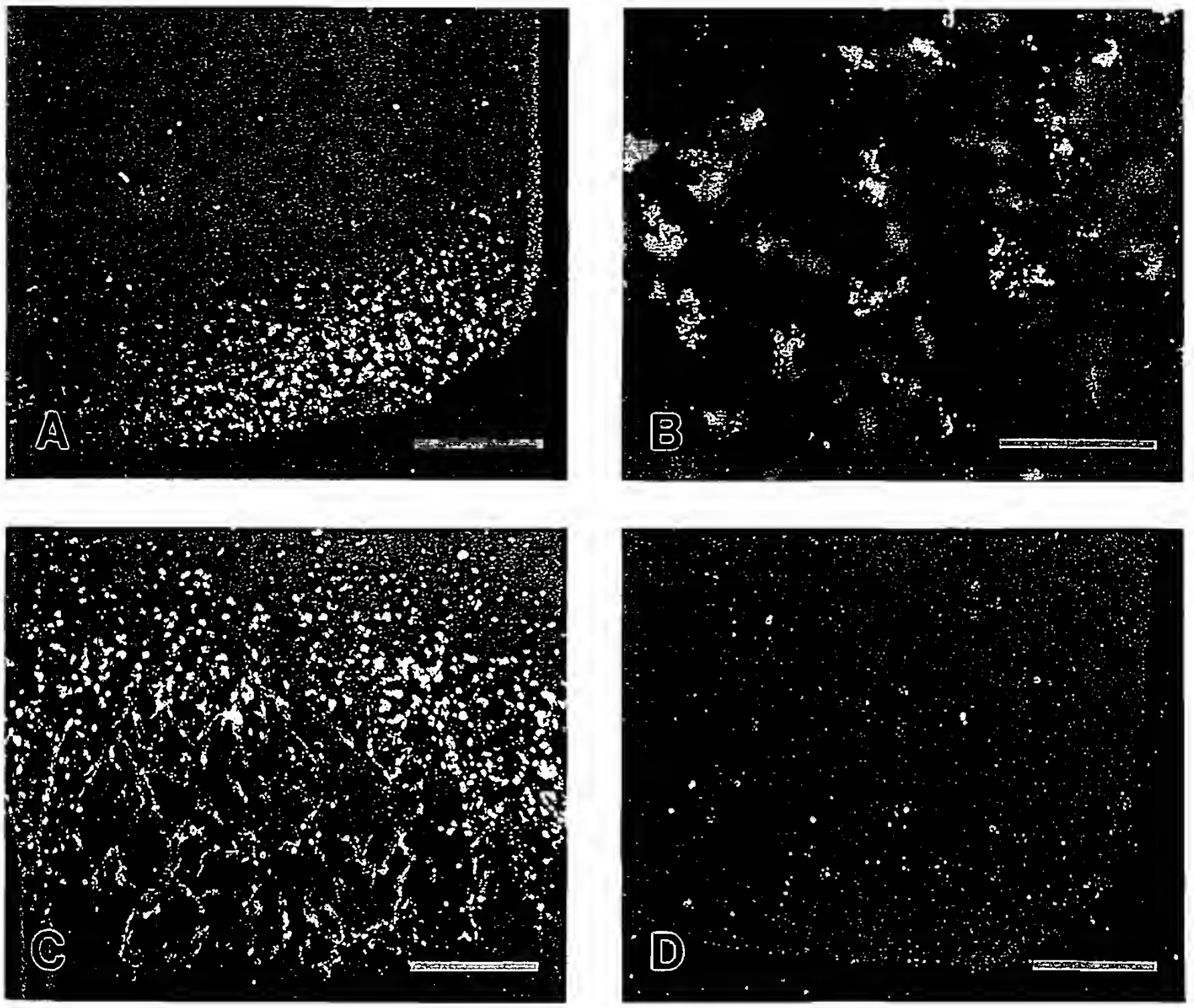

Fig. 8. The distribution of horseradish peroxidase (HRP), given 10 minutes prior to sacrifice, in the normal brain. Darkfield optics. The median eminence (**A, B**) and subfornical organ (**C**), regions considered outside the blood-brain barrier, are intensely labeled with HRP. **D:** No staining is noted in control sections of the median eminence that have been blocked with hydrogen peroxide. Bars = 200 μm in A, C, and D, 50 μm in B.

brain barrier to IgG, albumin, and HRP. We hypothesized that if perfusion fixation damages blood vessels, then similar patterns of permeability to each of these proteins should be apparent. In one experiment, IgG and albumin were immunolocalized in adjacent sections in the same animal. IgG was more widely distributed throughout the brain as compared to albumin. In addition, the distinct perivascular localization of IgG was not noted in adjacent sections immunostained for albumin.

In a second complementary experiment, we compared the distribution of IgG to HRP, given intravenously 10 minutes prior to perfusion fixation. We found that although vessels appeared impermeable to HRP, IgG was localized in a perivascular pattern as previously described. Taken together, these findings suggest that inadequate or uncontrolled fixation procedures were not responsible for the consistent perivascular immunostaining for IgG that was observed in our studies.

The distinct perivascular localization of IgG raises an important question regarding the source(s) and pathways(s) of IgG in the brain. At the ultrastructural level, the protein was localized in the Virchow-Robin spaces, along the basal lamina of microvasculature, and in the adjacent parenchyma. Based upon this study as well as those of other investigators (Brightman, 1968; Rennels et al., 1985; Broadwell and Sofroniew, 1993) we can only speculate on the routes by which IgG has access to the brain. Broadwell and Sofroniew (1993) suggest that plasma proteins access the brain along vascular sites known to be void of blood-brain barrier properties. These include blood vessels associated with circumventricular organs and the subarachnoid space. From these sites, protein may then enter the cerebrospinal fluid (CSF) and/or extracellular compartments of the brain. CSF-borne macromolecules may traverse the ependyma to enter the extracellular space or be routed along the Virchow-Robin spaces (Brightman, 1968). The latter path-

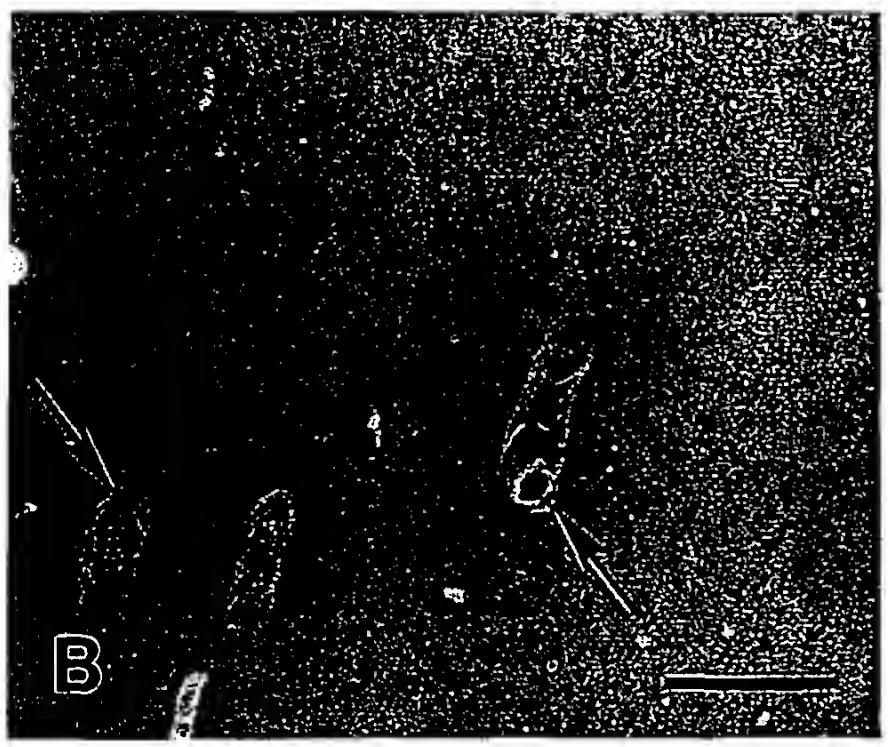

Fig. 9. The typical perivascular distribution of IgG as compared to HRP in the hippocampus. Darkfield optics. **A:** HRP is not apparent in the hippocampus. As a result, vessels appear as dark silhouettes against the background illumination (arrows). **B:** In contrast, vessels are intensely labeled with Pab IgG (0.03 mg/ml, arrows). Bars = 200 μm.

way has been shown to serve as an effective corridor into the brain. Rennels et al. (1985) noted that HRP, infused into the lateral ventricles or subarachnoid space, gained access, within minutes, to the extracellular compartment of the brain. Dense deposits of reaction product were detected in a cuff-like periarteriolar distribution. Labeling was also associated with venules and veins when the time course for exposure to the tracer was extended.

Although it is possible that the perivascular route (subarachnoid space) contributes to the pattern of immunostaining, in this study we suggest that IgG may also have access to the extracellular space directly across intraparenchymal vasculature. This hypothesis is based on the observation that perivascular staining was patchy in its distribution along the vascular tree. If the Virchow-Robin spaces were the primary routes for IgG entry into the brain, then one might expect a more uniform pattern of perivascular staining that would preferentially delineate arterioles.

Several indirect lines of evidence support the view that brain vasculature expresses a limited ability to transport IgG. Recent studies have demonstrated a close anatomical association between perivascular immunolabel and intrinsic brain vessels. Fishman and Savitt (1989) immunolocalized IgG in microglia. Although the source of IgG was not studied, these investigators noted a close association between immunolabeled microglial processes and microvasculature. More recently, Schmidt-Kastner et al. (1990) examined the permeability of hippocampal vasculature to IgG and albumin. These investigators reported that approximately half of the vessels in the hippocampal fissure exhibited perivascular immunolabeling for these proteins. Taken together, these studies provide indirect evidence for microvascular permeability to IgG.

Several studies have provided more direct evidence for transport of circulating plasma proteins across brain microvessels. Westergaard (1980) and Westergaard and Brightman (1973) examined the permeability of the blood-brain barrier to HRP and ferritin at the ultrastructural level. Cerebral arterioles, joined by tight junctions, exhibited permeability to these vascular tracers. It was postulated that protein was transferred across the endothelial cell in membrane-bound vesicles.

A similar transport system may accommodate blood-brain transfer of IgG. Zlokovic et al. (1990) evaluated the transport of IgG across the blood-brain barrier of the rodent. Brains were exposed to a perfusion medium followed by isotopically labeled ^{125}I-IgG in the presence and absence of unlabeled IgG. In complementary studies, IgG was also immunolocalized in the brain after perfusion with unlabeled IgG. The kinetic studies demonstrated the presence of a transport mechanism for IgG that was saturated at normal physiological levels. Furthermore, the protein was immunolocalized both within endothelial cells and in the adjacent perivascular parenchyma. Triguero et al. (1989) evaluated the unidirectional transport of ^{125}I-labeled IgG during a single transit through the rodent brain. They reported 3.5–5.2% of the labeled IgG was extracted during this interval. Although this is a relatively limited quantity, it is important to emphasize that this value was determined 5 seconds after administration of the labeled protein. In summary, ultrastructural, kinetic, and immunocytochemical studies support the view that the blood-brain barrier to IgG under normal conditions is not absolute.

In the present study, we noted that IgG was consistently immunolocalized in distinct perivascular zones, but albumin was not. This finding suggests that the route(s) of entry for IgG into the brain may exhibit some degree of selectivity. Although this selectivity may be related to the immunologic nature of IgG, it is unlikely that it is mediated through the Fc receptors on cerebral microvasculature.

Fc receptors are present in various tissues of the central nervous system (CNS), particularly in regions outside of the blood-brain barrier and in microglia and macrophages, but they do not appear to be present on the surface of normal endothelial cells (Nyland and Nilsen, 1982; Perry et al., 1985). Nag and Gupta (1981) demonstrated surface Fc receptors on endothelial cells in vitro, yet Hughes and Lantos (1986) were unable to replicate this finding. They did, however, immunostain internal Fc receptors after permeabilization of endothelial cells. These internal receptors, which appear to be associated with cytoskeletal ele-

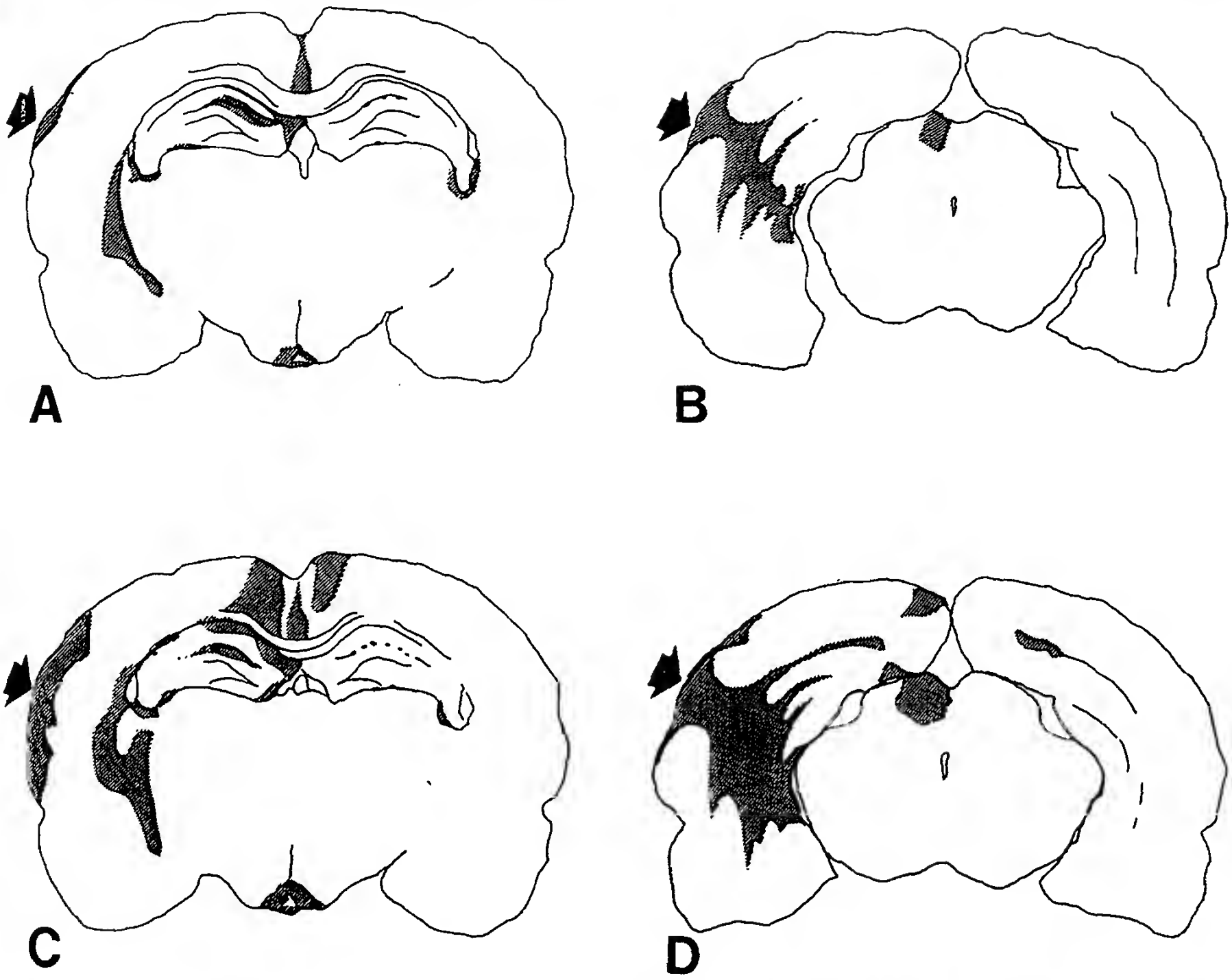

Fig. 10. Immunolocalization of Pab IgG and Mab IgG2a at 24 hours after traumatic brain injury. These tracings, drawn from adjacent sections of brain, demonstrate a close association between the distribution of Mab IgG2a (A, B, 0.001 mg/ml) and Pab IgG (C, D, 0.03 mg/ml) as defined by the hatched regions. As might be expected, Mab IgG2a exhibits a more limited distribution as compared to Pab IgG. Arrows indicate the site of impact.

ments, may play a role in the removal of damaged cells (Hansson et al., 1984); the mechanism, however, is unclear (Hughes and Lantos, 1986). Given the likelihood that IgG does not have access to these internal receptors under normal conditions, the mechanism for selective transfer of IgG remains unclear.

In this study, both IgG and albumin were immunolocalized in neurons in a diffuse pattern in the cytoplasm. Kozlowski et al. (1992) have noted a similar pattern of neuronal staining. The diffuse pattern of inmunostaining raises a question about neuronal integrity. It has been postulated that diffuse cytoplasmic distribution of serum protein may reflect indiscriminate entry across a damaged plasma membrane (Klatzo, 1967), whereas a punctate distribution in the cytoplasm may be indicative of a cell undergoing pinocytosis. We suggest that such an interpretation may not be applicable to the present study because of several technical considerations. First, given the thickness of the sections, it might be very difficult to discern a more localized, punctate distribution of the proteins. We noted, for example, a similar diffuse immunolocalization in brainstem neurons whose axons project into the periphery. These neurons are thought to participate in endocytosis and retrograde axonal transport of plasma proteins (Kristensson et al., 1971; Broadwell and Brightman, 1976; Sparrow, 1981; Balin et al., 1986; Fabian and Petroff, 1987; Yamamoto et al., 1987; Moos et al., 1991). The absence of a more punctate distribution in these cell bodies supports our hypothesis that the thickness of the sections limits cellular resolution. Second, others' interpretation of intracellular localization of proteins has been based primarily upon fluorescent probes and horseradish peroxidase. These markers may be detected with a higher degree of precision than is possible for IgG. In this study, the detection of IgG is based upon biotinylated probes that serve to amplify the signal but may also reduce the precision of localization at the light microscopic level.

Given these limitations, we can only speculate on the mechanism(s) by which IgG may enter neurons. As previously discussed, IgG may enter an injured neuron whose

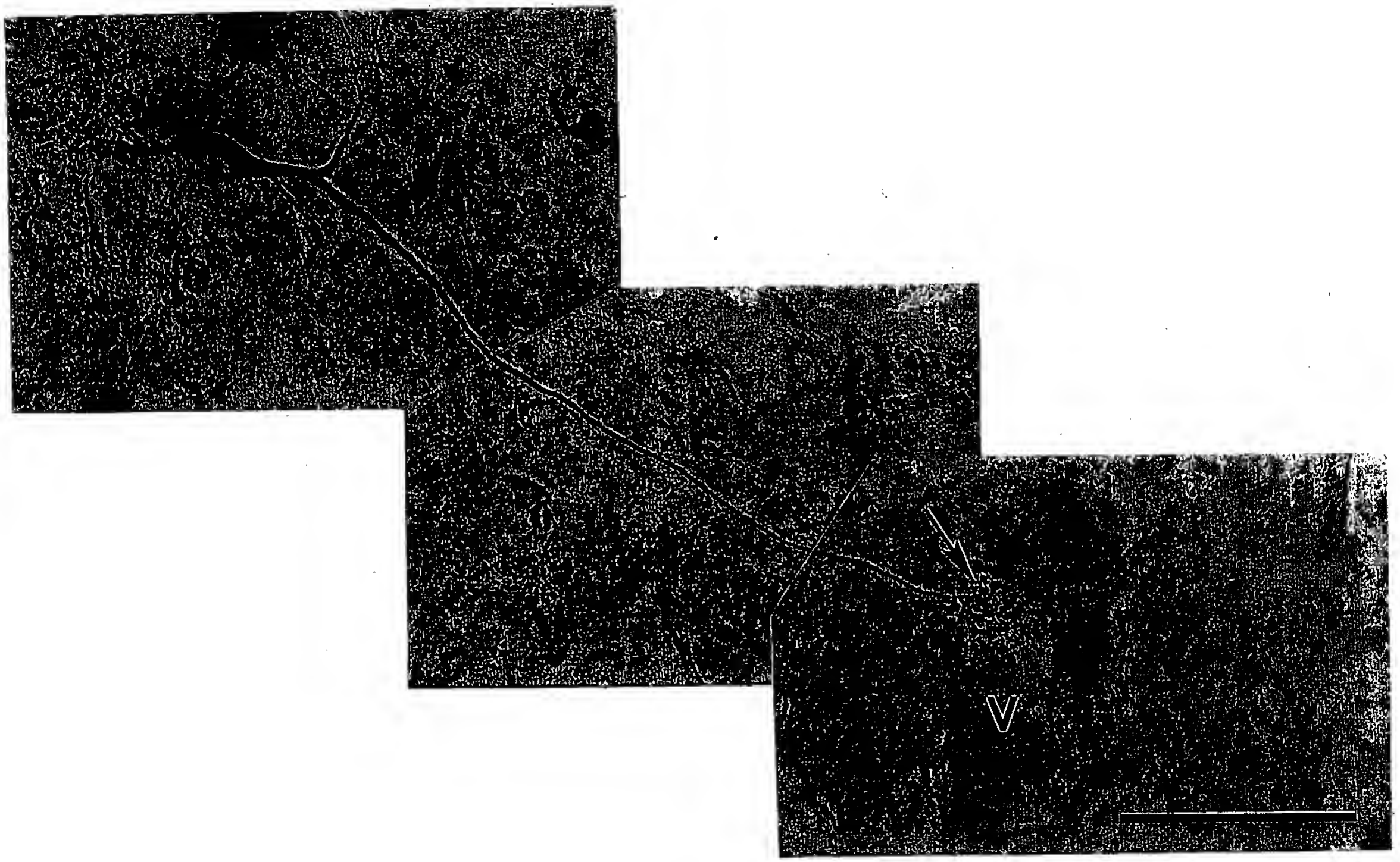

Fig. 11. Neuronal localization of IgG after brain trauma. Pab IgG (0.03 mg/ml) is immunolocalized in a large neuron in the superior colliculus. Immunolabeled parenchymal exudates are apparent within the dendritic field (**upper left corner**) as well as around the axon terminal (**lower right corner**). An immunolabeled process extends approximately 0.3 mm and appears to terminate near a blood vessel (V) that exhibits distinctive perivascular immunolabel (arrow). Bar = 100 μm.

plasma membrane is no longer intact. This may occur in those neurons located at the site of impact, where the barrier has become permeable to IgG. Thus, injured neurons may act as a sink for extravasated plasma proteins (Brightman et al., 1970). IgG may cross the plasma membrane, which has expressed a transient change in its fluidity. Povlishock et al. (1979) reported neuronal inundation with HRP after relatively mild head injury. These investigators suggested that such an appearance may reflect a transient, reversible state. This interpretation was based in part upon the absence of obvious ultrastructural alterations in labeled neurons. In the present study, it is conceivable that neurons, exposed to plasma components, express a similar transiently permeable state. Finally, neuronal somata and their processes may pinocytose IgG. This mechanism has been clearly demonstrated in neurons whose axons project into the periphery (Kristensson et al., 1971; Broadwell and Brightman, 1976; Sparrow, 1981; Fabian and Petroff, 1987; Yamamoto et al., 1987). The endocytosed protein may be directed to other parts of the cell by retrograde and/or anterograde transport (Tengvar, 1986). We believe a similar mechanism may occur since we observed immunolabeled neurons whose cell bodies or processes were in close proximity to immunolabeled perivascular sites.

Our findings would indicate that antigens and/or IgG may enter or exit the CNS while the blood-brain barrier remains functionally intact. The clinical relevance of such permeability is exemplified in pathologic states involving non-CNS tissues. For example, proteins generated by peripheral pathogens or cancerous tissue may utilize the same or similar route(s) to gain access to the CNS. This might facilitate the onset of autoimmune paraneoplastic syndromes, which occur when the CSF and/or CNS parenchyma present antigens shared with tumor tissues (Damau et al., 1992); how these antigens enter the CNS and why some enter more easily than others is not well understood (Pollack and Lund, 1990).

In summary, these immunocytochemical studies demonstrate the following findings: (1) IgG exhibits a distinct perivascular localization in the brain; (2) this perivascular immunoreactivity appears to be randomly distributed throughout the brain; (3) at the ultrastructural level, reaction product is closely associated with the basal lamina of capillaries, arterioles and venules and is also present in the Virchow-Robin spaces; (4) albumin does not exhibit the distinctive perivascular localization described for IgG, which suggests that the route of entry for plasma proteins exhibits some degree of selectivity; and (5) IgG is immunolocalized in neurons and glia. The close anatomical association between immunolabeled neuronal processes and perivascular immunolabel suggests neuronal uptake and transport of the protein.

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